

Too much caution on global warming

The much awaited Evans report in the United States is a disappointment, chiefly because it says too little about the prospect of global warming caused by the greenhouse effect. And because it says too little generally.

SLIM volumes are none the worse for not being encyclopaedic, and may well be more widely read. Yet there is a sense in which the much-awaited report on greenhouse warming published last week by the National Research Council in Washington could judiciously have been weightier. The Evans report, as the document has been called (after its chairman, previously a US Senator from the State of Washington), to which people have been looking for a resolution of the US government's doubts on global warming, is brief to the point of being laconic, even delphic. And it is late: a few months ago, people were hoping it would see the light of day before last February's climate conference in Washington. Has the extra time been spent winking out the occasional sentences that might have given offence to one side or another in what has become a polarized debate?

In the event, the report comes to all the right conclusions, but undemonstratively and with such an air of studied judiciousness that even the middle-of-the-road position that it adopts will not necessarily seem compelling to its readers. Indeed, the most striking and potentially controversial of its recommendations is that there is such a close link between the growth of the world's population and the future potential for global warming occasioned by the greenhouse effect that the "United States should resume full participation in international programs to slow population growth" and should contribute financially towards their cost. It is now roughly a decade since, early in the Reagan presidency, the US government declined to support most international birth-control programmes on the grounds that many of them held abortion to be a birth control method of the last resort. The case for participation is of course compelling, but has Evans reason to believe that the circumstances have changed?

On the more immediate international question, that of the clamour now under way for an international treaty to limit the emission of greenhouse gases, the relevant Evans recommendation is simply that the United States should "participate fully with officials at an appropriate level" in the discussions now under way, "including diplomatic conventions and research and development efforts". But the body of the report, referring to the plan to sign a treaty by June next year, adds the more prescriptive sentence that "the panel believes the United States should fully participate in this process". Hardly fighting talk.

For the United States in particular, the decision that lies ahead about the global warming treaty is at once more complicated and more interesting. There are arguments on either

side. There is, for example, something in the general suspicion that some would-be participants in a global warming treaty regard it all as a way of diverting yet more of the prosperity of the United States in their direction. On the other side of that coin, there are practical and almost incalculable considerations of the costs that may arise domestically — the huge amounts of capital that will be needed to maintain supplies of irrigation water at the level US farmers have come to expect. It will be strange if the nation boasting the most substantial research programme in the field stands aside from an international agreement from the fear of what happens to US governments seeking to control the demand for, say, gasoline by increased taxes.

Less tangible arguments also deserved a place in the Evans report. For example, as things are, the United States would be well-placed to ensure that next year's treaty is one that it could live with. So many of the critics of its recent display of unwillingness would be so quickly disarmed by a decision to join that they would sign whatever piece of paper the United States offered. (There are other reasons why the best agreement at this stage would be an agreement to act when research had shown that the time has come to do so, and in what way.) And as things are, the United States is well-placed to shame other recalcitrants, from Saudi Arabia to the Soviet Union, to put their weight behind a treaty. A cool discussion of these and other issues by a committee as influential as that of Evans might have had the scales falling from many pairs of US eyes. The document is an opportunity missed. □

Closing Europe's farms

The latest impending crisis in European agriculture argues for getting people off the land, and quickly.

PROSPECTS for a settlement of the long-standing dispute on agriculture between Europe and the rest of the world are fading fast, to judge from the commotion in Brussels in the past few weeks. By now, the story is familiar. Four years of negotiations towards an improved agreement on international trade under the General Agreement on Tariffs and Trade (GATT) broke down last December. By February, it seemed as if life might be breathed into the corpse of all that work by a change of policy at the European Communities (EC) headquarters in Brussels. But now it emerges that the members of the EC are unable to agree among themselves on how the



modest proposal canvassed in February might be implemented, while the gruesome prospect has arisen that Europe's farmers have responded to the deliberate reduction of agricultural prices within the European Common Agricultural Policy by further increasing their production.

Nobody should be surprised. Faced with reduced prices and offered a guaranteed market for what they produce, farmers have every incentive to maintain their cash incomes by increasing production. Even if, on a strict calculation allowing for such things as the cost of land, some of this production must be unprofitable, farmers have no urgent need to make the calculation, at least while they cannot think of what to do with their land except to grow unwanted food. But the extra and unbudgeted cost of buying up and storing the extra surpluses will have dire consequences in Brussels. There is a danger that the EC will not be able to afford the other good works on which it has set its heart because of the mounting cost of the surpluses. Ironically, it may not even be able to pay for the schemes being canvassed for winning a long-term contraction of the agricultural industry by paying farmers to take land out of production. Almost certainly, as matters have turned out, there will be no stomach in Brussels for the single step that would decisively unblock the GATT negotiations — a decision not to subsidize the export of surplus produce. And the opportunity to make a commercial bridge with Eastern Europe by encouraging low-cost production there will be denied, as will be similar and long-standing opportunities in the developing world.

That is why Europe, which is fond of hailing 1993 as the year in which it will become a true free market, needs a long-term plan that will be understood in Europe and elsewhere as a blueprint for a free market in agriculture. How is that to be achieved? What most clearly stands out is that occasional tinkering with the Common Agricultural Policy, or annual beastliness of fixing agricultural prices by the EC, will not suffice. Nor, in the long run, will schemes such as those now being canvassed to concentrate subsidies on certain classes of farmers — those with only a little land, for example. Many in Brussels and elsewhere rightly complain at such devices because they smack of social engineering (keeping small farmers on the land, for example), but all too often the discontent is directed more at the kind of social engineering implied than at the fact of it.

The plain truth would turn the intended reorganization of the Common Agricultural Policy on its head. Europe in the long run will more quickly become a more prosperous place in which to live if it has a low-cost source of agricultural produce, which means that it must find a place in an international free market in food. If there remain indigenous producers, they would naturally be the most efficient producers. That in turn implies that inefficient farms, which are often but not always small farms, should go out of business. But does that not imply that the farming tradition will be lost throughout Europe, and that whole rural communities will be impoverished? Those were the fears that made the Common Agricultural Policy a central part of the Treaty of Rome, which founded the EC. But they are out of date.

What Europe's agriculture needs is what European steel

and coal industries have enjoyed in past decades — institutional arrangements for helping communities of people dependent on one industry to make the transition to another, which may be very different. There are several strategems that might be followed, of which the most obvious (and not that slow) is to ensure that rural communities throughout Europe have access to first class secondary and higher education: too many of today's farmers farm because they know of nothing else to do. But there is also a wealth of experience to show that the alternative to farming is not the present blend of forestry and rural industry (from running half-empty hotels to the carving of wooden spoons). The countryside has traditionally attracted old-fashioned software manufacturers (authors and the like). With communications what they are becoming, and the demand for software growing also, it is not unreasonable to think of Europe's countryside as where its thinking might also be done. □

The Russian academy

A new venture in Moscow deserves support for what it may become, not what it is.

THE emergence of nationalism in the Soviet Union has been, for the past three years, one of the most serious threats to its continued administrative and political existence. The Republic of Georgia is the latest to have declared independence from the union, although it is by no means clear how it will be effected. By comparison, the efforts of a group in Moscow to create a Russian academy of sciences (page 547) may not be such heady stuff, but they are nevertheless significant.

The opportunity is the anomaly built into the Soviet system by the decision that there should be an academy of science in each of the republics, each in some degree dependent on the Academy of Sciences of the USSR, except for the Russian Federation, in which the main academy would hold sway. (Ironically, if the main academy had stuck to its instincts in the early 1930s, and had insisted on staying in Leningrad, Stalin would no doubt have created an All-Union Academy in Moscow and there would have been no problem.) The Russian parliament's declaration of autonomy last year — so far, it has not gone much further than that — was the spur for the decision to create a true Russian academy. The body that has sprung into being in Moscow is not for the time being an academy in the Stalinist mould — there is no network of research institutes, for example — and it does not even have a budget. But it is an interesting departure.

What can such a grouping do? The objective seems to be to create an organization to which people would be elected on the strength of their distinction as scientists, much as academies are organized elsewhere. It is a laudable ambition, but difficult to implement: people of distinction are chary of throwing in their lot with an untried organization, for example. But what such an organization could splendidly accomplish is to organize collaboration with people and laboratories elsewhere while winning its spurs for grander ventures. In that regard, it deserves a fair wind. □

Storm clouds gather over research costs

■ Will Congress put a cap on indirect costs?

■ Universities to conduct own audits

Washington

As the fallout of the US indirect costs scandal threatens a growing number of research universities, Congress and the White House are scrambling to rewrite the rules of science funding and to avoid such embarrassments in future.

Bruised by revelations that some schools may have improperly charged the government for millions of dollars of overheads in their research costs, both the executive and the legislative branch are considering radical changes, including possible caps or cuts in overhead levels. Last week, at a series of congressional and government hearings, officials and politicians disclosed half-a-dozen different reform initiatives.

The issue arose out of hearings that Representative John Dingell held into indirect cost charges at Stanford University where, he revealed, costs for a sail boat and for items in the university president's house had been allocated to research overhead. The situation took on a life of its own when Stanford chose to defend many of its allocations as legal under complex accounting rules, even though common sense said they have nothing to do with research.

Now, research universities across the country are taking a quick look at their own indirect cost records in anticipation of extensive federal audits. Many of the universities say they are unfairly tarnished by Stanford's brush and hope to prove that their indirect cost charges are legitimate. Nonetheless, many agree that it is high time to review the regulations. The question is how far revision of the rules will go.

Among the more worrying signs are hints that the House of Representatives Appropriations Committee may put a ceiling on indirect cost rates when it makes up the 1992 budget of the National Institutes of Health (NIH) in June. Last year, the committee capped agricultural research overhead at 14 per cent (see *Nature* 348, 184; 1990). By comparison, indirect costs in biomedical research range from about 60 per cent of direct research costs to nearly 100 per cent — one dollar to overhead for each dollar to science. Under indirect cost reimbursement, universities get federal funds to support libraries, administrative costs and basic services such as heat and light. Perhaps most important, the indirect cost pool, which for NIH totals about \$2,000 million, has become a *de facto* source of funds for new buildings and laboratory renovation.

At a hearing last week, William Natcher (Democrat, Kentucky), chairman of the House Appropriations subcommittee that

sets the NIH budget, asked NIH officials what they would think of an across-the-board cap. Although the officials responded by strongly discouraging the move, the suggestion sent tremors throughout the university community, which fears that the committee may use the Stanford scandal as an excuse to cut as much as \$250 million out of the NIH budget. A congressional staff member says that while it is too early to consider such draconian measures, "people should not ignore the fact that these questions are coming up more often".

In another telling development, the White House Office of Management and Budget (OMB) last week broke off negotiations with the Association of American Universities (AAU) on new indirect cost accounting methods. The two had been working since last year to implement the recommendations of an 1988 AAU panel headed by Cornelius Pings, provost of the University of California. While the Pings report had proposed a more uniform accounting method than the present arcane system, the recent controversy has convinced OMB to expand the reforms even further, towards eliminating all items that can not be obviously linked to research, even if they can legally be charged to overhead under current practices.

In other developments in the indirect cost row last week:

■ Harvard Medical School withdrew about \$500,000 in indirect costs that had been "mistakenly" billed as research overhead after an outside audit conducted by a private accounting firm. In addition, the medical school, which has argued for an indirect cost rate of 104 per cent, now says it will temporarily lower its requested rate to 65 per cent.

■ The Department of Health and Human Services announced that it is expanding its probe of indirect cost charges at major US universities from four to about 20 schools.

■ One of them, Johns Hopkins University, like the others on the list, has begun its own audit in tandem with that by federal officials. According to a statement from Hopkins officials, the university has always opted to exclude from its indirect cost pool items such as maintenance of the president's house and administrative costs of such offices as public relations and community affairs.

■ Representative George Brown (Democrat, California), chairman of the House Science Committee, told reporters that he was working with the White House Office of Science and Technology Policy (OSTP) on a mechanism to protect research facilities from cuts in indirect costs.

Christopher Anderson

FETAL TISSUE RESEARCH

Tissue ban under fire

Washington

BERNADINE HEALY, the new director of the National Institutes of Health (NIH) and the official charged with enforcing the Administration's controversial ban on fetal tissue research, faced a strong challenge from the liberal chairman of the House subcommittee on health and an outspoken opponent of the ban, Representative Henry Waxman (Democrat, New York), at a congressional hearing early this week.

The two played out their respective roles with aplomb, as Waxman orchestrated a four-hour attack on the ban, while Healy neatly sidestepped the most difficult questions.

Waxman managed to dig up two perfect witnesses — a Baptist pastor and his wife — who despite fervent anti-abortion sentiment, had saved the life one of their children with a rare genetic disease (mucopolysaccharidosis type I) by allowing researchers to transplant fetal tissue into the child.

In rationalizing the use of bone-marrow tissues taken from an aborted embryo, the couple searched scripture, eventually finding reference to God making Eve out of Adam's rib. "It was clear to us that God was not opposed to tissue transplants," Rev. Guy Walden explained to the committee.

Turning to Healy, Waxman zeroed in on her explanation of the role of science in the political debate over fetal tissue.

Although Healy had voted to support fetal tissue research three years ago when she served on the NIH panel on the subject, she has been forced to defend the Administration ban since her nomination as NIH director last year.

"Sometimes science has to take a 'time-out' when its goals collide with the moral and ethical concerns of society", she said.

"When can we expect this 'time-out' to be over? Waxman asked.

Healy responded: "I view it as a 'time-out' in the sense that science is an evolving story. As more information comes in, it will be made available to the Department [of Health and Human Services (HHS), which is the parent agency for NIH and, in the person of conservative assistant secretary for health James Mason, the locus of the ban]."

In other words, she hopes to work from within to convince HHS of the error of its ways. Meanwhile, Healy said that NIH continues to do fetal tissue research that does not involve transplantation, and to study the broad area of human transplantation of non-fetal cell lines.

"The ban is razor sharp," she said. "Dr Mason wants to avoid the one-to-one relationship between abortion and therapy. But outside of transplant [of fetal tissue] research, the science is moving on."

Christopher Anderson

Survey pans university labs

Tokyo

TENS of thousands of professors and researchers at Japan's national universities have produced a damning assessment of the standard of research facilities in their institutions, in a survey just released by the Association of National Universities.

The survey comes at a time when Japan's principal science policy-making body, the Council of Science and Technology (CST), chaired by the Prime Minister, is debating problems in Japan's public-sector research environment. Given this timing, the survey will undoubtedly influence the council's recommendations later this year, which will determine Japan's public sector science policy for many years to come.

To assess the state of research in Japan's universities, the university association sent a questionnaire to 53,248 professors, associate professors, lecturers and research assistants at the country's 96 national universities in December last year. Of the 65 per cent (34,325) who replied, about 85 per cent said that facilities in private-sector industrial research institutes were "much better" (60 per cent) or "slightly better" (25 per cent) than in their own universities. Similarly, they rated their universities poorly compared with private universities.

Many those working in science, engineering, medical, dental and pharmaceutical science departments complained that they have to use their own money to pay for domestic and international travel, books for their libraries and equipment, because funds from the Ministry of Education, Science and Culture (MESC), which is responsible for running the universities, are inadequate. The average personal outlay for these items each year was ¥570,000 (\$4,200) in science, engineering and agriculture departments, and ¥870,000 (\$6,400) in medical, dental and pharmaceutical science departments.

Seventy-five to eighty per cent of respondents said that analytical equipment, reagents and computer and information processing facilities are "totally inadequate" or "inadequate". In addition, funds for general expenses (such as telecommunication bills and salaries for assistants) were found to be two-and-a-half to four times less than necessary, and the number of competitive research grants available from MESC was called inadequate.

The survey should provide ammunition for those members of CST pushing for drastic reform of public-sector research and, in particular, the national universities. A sub-panel of the council, which is composed of academics, industrialists and government officials, recently began hearings on a new set of recommendations that will set policy for the next five to ten years. (The last such recommendations were made in 1984 and were adopted by the cabinet in 1985).

Previous council recommendations have

tended to be rather anaemic calls for improvement of basic research that have failed to get to grips with the problems in the national universities. One such problem, for instance, is that while CST is supposed to set policy for all the science-related ministries, its secretariat is located in the Science and Technology Agency (STA). As a result, the council tends to be regarded with suspicion by MESC, a bitter rival of STA, and in previous CST recommendations all references to national universities have been deleted at the request of MESC officials, who insist that such matters lie in the domain of the ministry's own science council.

But this time around, the pressure to do something (and to say something) about the universities is enormous. Japanese industry is becoming very concerned about the tendency of young Japanese to shun careers in science and engineering in favour of jobs in finance and economics. Industrialists and the Ministry of International Trade and Industry (MITI) say that unless something is done quickly to attract young people back to science and technology in the universities, Japan is headed for almost certain economic decline.

In the past, MITI has not paid much attention to CST deliberations. But this time a member of the ministry has been assigned full-time to work with the council in STA.

MESC is most unlikely to solve the problems on its own. One former senior official of Tokyo University points out that power (and budget) in MESC is concentrated in the sections that deal with high-school education, and that the sections that deal with research at the graduate level and above have comparatively little power to influence the ministry's policy and allocation of funds.

MESC officials dealing with research have tried to cope with this situation by expanding the budget for competitive grants while holding down general research funds that automatically go to all faculty. The intention is to cut off funds to those not actively involved in research.

Government officials outside MESC and the university researchers recently polled realize that it is probable that the only solution lies beyond the realms of MESC. Seventy-two per cent of respondents suggested that extra funds should be obtained from other ministries, 65 per cent suggested local governments, 68 per cent suggested private foundations funded by Japanese industry and 54 per cent suggested private industry itself.

MITI officials say that a foundation funded by Japanese industry and perhaps affiliated with STA, MITI and MESC might be an acceptable solution. But much depends on whether CST will finally decide to say something about Japan's universities when it issues its recommendations towards the end of this year.

David Swinbanks

New channel is lost in space

Tokyo

A COMBINATION of recent solar flare activity, the eccentric orbit of the Earth around the Sun, and a faulty solar panel seem likely to wipe out broadcasts of at least one of three new satellite television channels watched by millions of Japanese.

Japan's National Space Development Agency (NASDA) announced last week (10 April) that it expects difficulties with its new broadcasting satellite, Yuri-3a, launched last August. The satellite, which has defective solar panels, will be unable to sustain broadcasts of Japan's three channels between mid-May and mid-August.

The news must be particularly distressing for about 300,000 Japanese who have just paid ¥27,000 (\$200) for decoders plus ¥2,000 a month to watch a new commercial satellite channel.

The 'WowWow' commercial channel began pay service at the beginning of this month. Before that, Japan Satellite Broadcasting Company (JSB), which operates WowWow, had broadcast unscrambled signals via Yuri-3a to attract subscribers.

NASDA officials blame the breakdown in part on what they call "unexpected" solar flare activity in March. Apparently, a shower of high-energy protons from the flares knocked out a few per cent of the panel's solar cells. And as the panels were already producing only about 75 per cent of their designed output (see *Nature* 347, 114; 1990), the additional loss was enough to reduce power below the minimum required for full services.

From mid-May to mid-August, the satellite will have enough power to broadcast on only two channels because during that period the Earth will be at its farthest point from the Sun in its elliptical orbit and also because the Earth's equatorial plane — in which Yuri-3a lies — will be at maximum inclination to the Sun. As a result, incidental sunlight on the satellite's solar panels will be less intense and the panels' power output will be reduced.

NASDA has suggested one possible solution. Yuri-3a's predecessor, Yuri-2b, which went out of service at the end of last year, could broadcast two channels for about a month before its fuel supply runs out. If JSB and NHK, the national broadcasting corporation that operates the other two channels, can agree to share the combined capacity of the two satellites, it may be possible for broadcasting services for the three channels to hobble along until another NHK broadcasting satellite (BS3H) due to be launched by a US Atlas rocket on Friday (19 April) comes into service at the end of May or the beginning of June. But NHK is reluctant to accept that idea until BS-3H is launched and fully tested.

David Swinbanks

HEALTH

Can lefties study be right?

Washington

A STARTLING study that found that left-handers die an average of nine years earlier than right-handers has triggered a sharp debate among psychologists and epidemiologists. Although most researchers are willing to accept the possibility that, for both physiological and environmental reasons, right-handers do indeed live longer than left-handers, few can believe that the difference in life expectancy could be so great.

"If you think about it, this is a bigger difference than if you smoke two packs a day," said Alan Searleman, a psychologist at St Lawrence University in Canton, New York, who has studied differences between right- and left-handed people. "If it were true, you'd think that over the years it would have been noticed."

But until this month, the only evidence of a disparity in life expectancies between the two groups had appeared three years ago in *Nature* (333, 213; 1988). In that report, psychologists Stanley Coren of the University of British Columbia and Diane Halpern of California State University had studied the

mune diseases and are more likely to have been born prematurely or to have been low-birth-weight babies.

Other work, including an earlier study by Coren, has suggested that left-handers are more likely to have accidents. The disparity in accident rates may arise, Halpern and Coren suggest, because life in an environment designed for right-handers may be slightly more hazardous for left-handers.

Coren and Halpern's paper has received extensive coverage in the US press, and has sparked concern over the possible public policy implications, if indeed the results are replicated. Would insurance companies, for instance, be justified in charging left-handers higher rates for medical and life insurance?

Meanwhile, various aspects of the Coren-Halpern study have been questioned. One

consistent observation is that natural left-handers growing up early in the twentieth century may have been coerced into using their right hands — which would make it appear now as if the older left-handers had died off, when in reality they had simply turned into right-handers. Coren argues, however, that several studies have shown that the percentage of adults who are left-handed has not varied since the early 1900s, but has remained at about 10 per cent of the population. Searleman, in response, says that those conclusions depend on "spooky data" and should not be trusted.

The best way to settle the issue, everyone agrees, would be to have a large prospective study following a large cohort for a decade or more, recording deaths and their causes. Such a study would be both expensive and time-consuming, but until it is done, the true risk of being left-handed will remain a subject of debate.

Robert Pool

SUB-SAHARAN AFRICA

Spotting famine from space

Washington

APRIL marks the beginning of the rainy season in the Sahel region of sub-Saharan Africa, and the start of another critical period in determining the future food prospects in this famine-prone area. Because of a poor harvest last year and a continuing civil war, Sudan is the most vulnerable country in the region.

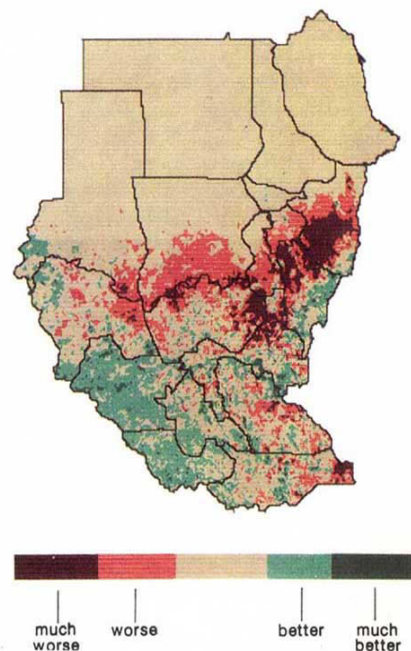
Now, a technological crystal ball is predicting that famine will probably strike that country hard. The prophecy is based on data gathered by the Famine Early Warning System (FEWS), a project that anticipates food needs in this region of Africa using satellite and on-site reports. FEWS, which has been operating since June 1989, tracks weather as well as political and economic conditions, and enables scientists to predict areas that may be badly hit in the coming season.

But hard experience teaches that prediction does not equal prevention. With all the advance warning and high technology of the FEWS system, food aid is still sometimes difficult to get into the countries that need it the most. For example, FEWS predicted last year that Sudan would be a trouble spot and mobilized food aid to avert disaster, but poor transportation and lack of government cooperation in the country severely hampered relief efforts. "The biggest problem in the Sudan is the government's not wanting to admit that there's a problem," says Marian Mitchell of the FEWS project. Conditions are expected to worsen, and up to nine million people are at risk of starvation.

A NASA Advanced Very High Resolution Radiometer satellite estimates the vegetation, or greenness of the ground, by scanning the Earth with infrared and near-infrared frequencies of light to gauge the amount of chlorophyll. Generally, Mitchell says, rain in an area shows up about 10–20

days later as in a greener landscape detectable by the satellite system.

Actual rainfall is measured at stations in each country, and the data are transmitted to FEWS every ten days. And field representatives monitor political and economic conditions in seven countries — Mauretania, Mali,

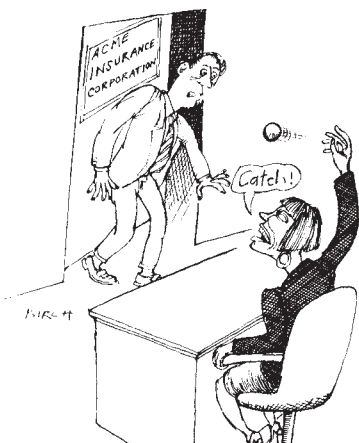


Drought conditions in Sudan as revealed by NASA's radiometer satellite.

Burkina, Niger, Chad, Sudan and Ethiopia.

By combining these data investigators can estimate rainfall levels and the potential consequences to food crops. The results are published by FEWS every 10 days during the rainy season, which alert officials of the US Agency for International Development to potential crisis areas.

Diana Steele



life expectancies of professional baseball players and discovered a difference of eight months in average lifetimes.

On 4 April, Coren and Halpern reported a much stronger result in the *New England Journal of Medicine* (324, 998; 1991). They had determined the handedness of 987 people who died in two southern California counties, defining as right-handed all those who wrote, drew and threw a ball with their right hands, while left-handers and mixed-handers were lumped together as 'non-right-handed'. When Coren and Halpern calculated that average age of death for the two groups, they found it to be 75 years for right-handers and 66 among the non-right-handers. The pattern held up when the cases were divided by sex: males 72 versus 62, and females 78 versus 73.

The idea that right-handers may, on average, live longer than left-handers is not particularly surprising, say those in the field. Previous studies have found, for instance, that left-handers are more prone to autoim-

Medicine becomes a liability

London

THE University of London's strength in medicine may bring financial disaster to its colleges, as government reforms to the National Health Service (NHS) and to research funding take effect, university officials say.

The university now attracts 40 per cent of the UK medical research charities' spending in the universities and its eight undergraduate medical schools train one third of Britain's new medicine doctors. But the government's plan to make hospitals operate like businesses, selling their services to local district health authorities, could eventually lead to London's expensive teaching hospitals losing patients, with some even sliding towards bankruptcy.

Under the NHS "internal market" introduced on 1 April, British hospitals must for the first time charge the full cost of the treatment they provide to the health authorities that send them patients. The health authorities have fixed budgets, and can choose to send their patients to the hospitals that offer the best value.

The problem for the University of London teaching hospitals is that their services are likely to be uncompetitive, as running a hospital in the centre of an expensive city increases costs. The worry is that health authorities that have sent patients into the centre of London may now turn to other hospitals in southeast England offering a cheaper service.

Sir Colin Dollery, dean of London's Royal Postgraduate Medical School and formerly chairman of the medical committee of the Universities Funding Council (UFC), fears that at least one of the London teaching hospitals could be critically short of patients within five years. The UFC might then have to think again about funding the medical schools linked to these hospitals, says Dollery, because a steady supply of patients is vital for teaching.

Many medical academics believe that the closure of some of London's medical schools is a good idea, if the students could be accommodated elsewhere. Sir Peter Swinnerton-Dyer, who stepped down as chief executive of the UFC last month, agrees that there is a case for shifting some medical education away from London. But he says he is worried that a patient- and cash-starved teaching hospital could do untold financial damage to one of London's colleges before any decisions are made — if, for instance, a college was tempted to pump more money into its medical school, in an attempt to maintain teaching standards.

Medical schools and teaching hospitals are, in theory, funded separately. But university-employed academics may spend much of the time treating patients, and NHS-employed hospital staff also help with teaching. The idea is that these factors balance out and that neither universities nor the NHS

lose financially.

But Derek Roberts, provost of University College London (UCL), which is linked to two teaching hospitals, believes that financial difficulties in the NHS have already caused UCL to lose out, because academics have been forced to take on more and more of the burden of caring for patients.

A second worry for the University of London is the burden of overhead costs associated with the huge number of projects at the university funded by the medical research charities.

From 1992–93, the government aims to transfer some £100 million a year to the research councils from the £800-million-plus research budget of the UFC, in order to cover the indirect costs (administrative costs, telephone bills, library costs and the like) of research council-funded projects in the universities. The goal is for the research councils to take over responsibility for all the indirect costs of their projects, apart from the salaries of permanent academic staff, and the costs of running university buildings.

But the money from the UFC has also been used to pay overheads on charity-funded projects. And Peter Griffiths, clerk to the court of the University of London, says the transfer of £100 million to the research councils will place a crippling financial burden on London, which receives a disproportionately high share of its research grant income from the charities.

The charities spend more on university medical research than the Medical Research Council. London, with its concentration of medical schools, received £63 million from the research charities in 1989–90, outstripping its £55 million income from the five research councils. Most universities receive far more from the research councils than from the charities.

When the transfer from the UFC to the research councils was first announced, the medical research charities feared that they would be forced to pick up the overhead bill for the projects they support (see *Nature* 343, 199; 1990).

But Diana Garnham, from the Association of Medical Research Charities (AMRC), says the AMRC is now satisfied that the indirect costs of charity-funded projects can be met from elsewhere in the UFC's research budget. London should distribute its research money more selectively, she says, so that the best departments — where most charity grants are awarded — receive the most UFC money.

But David Bowles, vice-provost of UCL, maintains that the AMRC's assessment is "arithmetically incorrect" in London's case.

Even now, UCL loses 37 pence for every pound it receives from the charities, says Bowles, because the funds earmarked by the UFC to cover research overheads are inadequate.

Peter Aldhous

French minister clears Zagury

Washington

AIDS researcher Daniel Zagury, who has conducted a variety of vaccine studies during the past couple of years, has been cleared of charges that he acted without the approval of the appropriate ethics committees in France. Allegations that Zagury injected candidate vaccines into people without the permission of ethics committees were levelled by reporter John Crewdson of the *Chicago Tribune*, who also accused US researchers whose materials Zagury used of failure to get proper approval from the US National Institutes of Health. That allegation is being investigated by an NIH ethics body which has not yet reported its findings.

However, as to Zagury's work in France, French authorities concluded that "legislative texts, procedures, and recommendations of ethical committees have been respected" and that Zagury's experiments were approved by the national government as well as the Hôpital Saint Antoine in Paris where he works.

Barbara J. Culliton

ZOOLOGICAL GARDENS

London's ark runs aground

London

THE fate of London Zoo remains in doubt after the Department of the Environment last week refused to underwrite a £30 million rescue package to avert the zoo's imminent closure and cover its £2 million annual deficit as well as some future development. The money would also have been used to carry out long-overdue maintenance of nine listed buildings at the zoo required by the terms of its lease, which is from the Department of the Environment.

The zoo is taking a pragmatic view of the rebuttal, however, and says it regards discussions with the government as ongoing. Another meeting is scheduled for the end of the month. The zoo is run by the Zoological Society of London, whose research branch, the Institute of Zoology, is effectively immune from the financial problems at the zoo. The institute received £1.3 million of government funding through the University of London in the financial year 1989–1990, plus £793,000 from various other research bodies.

Meanwhile, speculation is rife about what will happen to the central London site and its 8,000 animals. A transformation into a centre for conservation education with the relocation of the larger animals to the more spacious Whipsnade Zoo, 35 miles away, has been debated. But rumours of a wholesale move of London Zoo itself are "a complete red herring", according to the zoo.

Henry Gee

"For science and Russia"

Moscow

THE Russian (as distinct from the Soviet) Academy of Sciences held its first general meeting last month, to confirm the election of over 400 members and alternate members. But the future of the institution is still uncertain: there is as yet no assured budget, while the academy has still to show itself a worthy counterpoise to the "big academy, the Academy of Sciences of the USSR."

The new academy has come into being, according to president Dmitry Mineev, "in the interests of science and Russia". Mineev, head of the mineralogy and geophysics department at the Moscow Institute of Geology and Prospecting, is the author of 300 scientific papers and the discoverer of eight phosphate and rare metal deposits on the Kola peninsula. Traditionally, Russia has differed from most other republics in not having an academy of its own; it was formerly held that the big academy covered its functions, although only 10 per cent of its full members are from Russia.

The new academy (abbreviated to RAEN) has had a hard birth. While RAEN's founders were engaged in heated debate over the principles of its constitution, the presidium of the big academy seized the opportunity by resolving to form its own Russian Academy and by appointing Nobel prize-winner physicist Alexander Prokhorov as its president. But the plan to form an academy "from above" was not to the liking of many working scientists. Prokhorov refused to assume the post and that project remains in limbo.

RAEN was formed by a group of universities and colleges, research centres and Soviet academy institutes. At its first conference last autumn, 140 full and alternate members were elected, which list has now been enlarged. The vice-presidents elected last month are biologist Nikolai Vorontsov, chairman of the USSR Environmental Protection Committee, physicist Sergei Kapitsa, president of the Soviet Physical Society and laboratory chief of the Vavilov Institute of Physical Problems, and geologist Oleg Kuznetsov, director of the All-Union Research Institute of Geological and Geophysical information Systems.

Unlike the big academy, RAEN will not limit its membership, so that it will not be necessary for deserving candidates to wait for a vacancy. Far from being paid for their fellowship, RAEN members pay dues — a symbolic 25 roubles a year. The academy has no subordinate agencies that might bring it funds, but intends to form and finance *ad hoc* research groups to carry out studies.

The budget problem seems likely to persist. The 600,000 roubles contributed by its founders is just a drop in the bucket. The founders intended the academy as a state-owned organization financed by the government of the Russian Federation. But the

Russian authorities see it as a public organization, which does not exclude budget financing of the particular themes and programmes commissioned by the Russian government, but which explains Mineev's calls for a search for other sources of finance.

RAEN has six sections: physics; chemistry; the Earth sciences; mathematics, informatics and cybernetics; biology and medicine; and Russian encyclopaedia. The last refers to a joint project with the organization known as Resurrection to compile and publish an ample encyclopaedia series dedicated to the history of the science and culture of people living on Russian territory. Among the planned volumes are encyclopaedias in

MEDITERRANEAN OIL SPILL

Riviera under threat

Turin

THE Italian and French authorities have declared a state of emergency, after the western Mediterranean — and with it the economically important tourist beaches of the Riviera — was threatened by the spillage of up to 147,000 tonnes (about 1 million barrels) of heavy Iranian crude oil.

The Cypriot-registered tanker *Haven* is now lying in some 70 metres of water a few kilometres off the coast at Genoa, with up to 100,000 tonnes of its cargo still on board. The ship sank at the weekend, after being crippled by a series of explosions. Italian en-

Russian science, the Russian army and the Russian fleet, Russian aristocracy, *The Churches of Russia* and a children's encyclopaedia. The academy's other projects include plans to publish several scientific and popular magazines.

Last month's general assembly also elected its first honorary members: Alexander Solzhenitsyn, Iosif Brodsky, Mstislav Rostropovich and Yehudi Menuhin, as well as 18 Nobel prizewinners who are either of Russian origin or the offspring of former subjects of the Russian empire — Wassily Leontief (Harvard), Ilya Prigogine (Brussels), Andre Lwoff (Institut Pasteur), Bernard Katz (London), Milton Friedman (Chicago) and so on. Raen hopes these contacts will lead to collaboration within the Russian diaspora.

Yuri Kanin

the Haven shifting and breaking up, but is feasible.

Italian efforts to contain the spill have so far won general approval. Domitilla Senni, from Greenpeace's office in Rome, applauded the decision to tow the tanker several kilometers nearer the shore, before allowing it to sink. The sea floor slopes sharply south of Genoa, making any salvage attempt further offshore difficult, if not impossible.

Ruffolo had been under pressure to move the vessel away from the Italian coast, but said that this would have been "irresponsible" — creating the danger of a more widespread, longer term incident.

Most of the spilled oil has burned, but a large slick was on Monday being held by containment booms over the sunken tanker, and oil released at the original explosion was reported to be coming ashore at Alassio, 100km to the west. The extent of the ecological and economic damage will depend on the weather over the coming weeks. As *Nature* went to press, conditions were calm, but strong winds would badly hamper the effort to contain and collect the oil.

Claude Millot, from the CNRS Centre d'Océanologie de Marseille, France, says that sea currents and prevailing winds will tend to carry any oil that escapes westwards along the Italian and French coasts.

Ruffolo said that he will be pressing for tighter European Communities (EC) controls over the transport of oil in the Mediterranean at a meeting of EC environment ministers on 30 June. But the fundamental problem, he said, is the heavy volume of oil traffic in and out of Italy's 30 oil terminals. Italy now relies on oil for almost 60 per cent of its energy needs.

Peter Aldhous

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

The view across Genoa harbour, Italy, last Saturday. (AP)

vironment minister Giorgio Ruffolo, in Turin on Monday to open a three-day meeting on Oceans, Climate and Man, organized by the Foundation San Paolo, said that underwater surveys were already underway to see if this oil can be salvaged, to prevent further spillage.

Pumping oil to a tanker at the surface will be a difficult operation, requiring the careful replacement of oil with sea water, to prevent

Times getting tough for an ex-giant

Jena, Germany

Just last month, it seemed as if Jenoptik Carl Zeiss JENA, the world-renowned eastern German optical company, would survive the rigours of German unification. Facing a takeover by its western German counterpart, Zeiss-Jena had been threatened with virtual shutdown and the scrapping of many of its lines of microscopes, telescopes, lasers and other scientific equipment. Then, on 6 March, the German *Land* of Thuringia (where Jena is located) stepped in and offered to take ownership of the company under a government-sanctioned agreement that would even absolve Zeiss-Jena of its DM1,350 million (about \$800 million) debt.

Now, however, it seems as if the news may have been too good to be true. After apparently taking a closer look at Zeiss-Jena's disastrous financial situation, Thuringia announced on 27 March that it needed more time to negotiate details of the agreement.

The *Land's* takeover of the company, originally scheduled for 1 April, has been postponed until at least 31 May, raising the possibility that Thuringia may not be able to rescue Zeiss-Jena after all.

For scientists, particularly those in developing countries and the former Eastern Bloc countries, the troubles of Zeiss-Jena have a special significance. In the West, the company has a solid reputation for making precision measuring devices and certain optical instruments such as microscopes, binoculars and aerial cameras — the latter being a field where the company commands a large part of the US market. And in the former Soviet bloc, Brazil and elsewhere in the developing world, Zeiss-Jena had a virtual monopoly on high-technology optical equipment. It has, for example, built more scientific telescopes in the one- to two-metre range than any other company in the world, producing 12 telescopes (mostly for institutes in the Soviet bloc) in the past 30 years.

But the success or failure of Zeiss-Jena will affect more than just the supply of certain types of scientific instruments. In many ways, the way in which the company's predicament is resolved will be a test case for eastern German industry in general. After all, the optical company has a respected reputation, a skilled workforce and a good product. If it cannot compete successfully in the free market, how can other international businesses in the former Communist country hope to do better?

Zeiss-Jena has a distinguished history going back to 1845, when Carl Zeiss founded his original microscope factory in Jena. For 100 years the company grew and prospered, but, at the end of the Second World War, it, along with Germany, was split in two. In June 1945, departing Allied forces took more than 100 executives and technicians out of Jena and settled them in a suburb of Stuttgart.

There the Zeiss employees founded Carl Zeiss-Oberkochen, which by 1989 had grown to 31,000 workers and net sales of DM4,500 million (\$3,000 million).

Zeiss-Jena prospered too during that time, but by a different path. Set up as a *Volkseigener Betrieb*, or people-owned company, it moved into producing cameras, eyeglasses and, ultimately, computer chips, in addition to its core business of microscopes, telescopes and other optical devices. By 1989, it had 70,000 workers and an annual turnover of 3,900 million East German marks (equivalent to \$2,600 million at the official — unrealistic — exchange rate).

Everything changed, however, with the unification of the two Germanies. The East German government had subsidized Zeiss-Jena — as it had many other exporting com-



Physicist Ernst Abbe, who shaped the company in its formative years.

panies — by guaranteeing that its products would be bought at unrealistic prices. When East Germany carried out a currency reform in July 1990, making two East German marks equal to one West German mark, Zeiss-Jena became unprofitable overnight. Suddenly the Soviet bloc, which had accounted for 65 per cent of the company's market, could no longer pay for Zeiss-Jena products because the company demanded hard currency instead of 'transfer roubles', the artificially priced money of the Soviet Union and its satellites. (Many Soviet and Eastern European scientists miss the old arrangement just as much as does Zeiss-Jena, because, now that they can no longer pay in roubles, their supply of optical equipment has dried up, with no replacement in sight.)

In response to the economic pressures, Zeiss-Jena has dropped its camera and chip sidelines and reduced its work-force from 70,000 to 27,000 workers. And the management has developed a blueprint for further rationalization, keeping just 10,000 workers.

Against this background, the two Zeiss

siblings began moving cautiously towards a merger. In May and November 1990, they signed agreements to work cooperatively toward reunifying Carl Zeiss. But when rumours circulated that the western German company would release all but 2,700 workers, the atmosphere turned unpleasant. On 13 February, 20,000 workers demonstrated in Jena, demanding job security.

Three weeks later, the *Land* of Thuringia offered to take over the company from the governmental trust body that had been set up by Bonn to refinance and stabilize eastern German industry and which had overseen the negotiations with Zeiss-Oberkochen. But Thuringia suddenly backed off — apparently when it realized that it could not pay for the company's continuing losses, which financial analysts estimate could eventually reach DM2,000 million.

So now reunification with Zeiss-Oberkochen has been given another chance. The trust body is conducting one more round of negotiations, and these include four parties: the two Zeiss companies, Thuringia and Baden-Württemberg, the *Land* in which Oberkochen is located.

But Zeiss-Jena is not just sitting back and waiting to see who will end up in control. Instead, it is scrambling to carve out a niche on the Western market, primarily with highly specialized projects that emphasize its scientific strengths. For example, it is building a mirror for an experimental six-legged optical telescope; the designers turned to Zeiss-Jena when they could not find a Western company to build the mirror to the necessary specifications (see *Nature* 341, 177; 1989).

In a project for the European Space Agency (ESA) technology centre in Noordwijk, the Netherlands, Zeiss-Jena is developing highly precise testing equipment for satellite-based lasers. The lasers will be used to transfer data from a satellite in a low-Earth orbit to a satellite in a higher, geostationary orbit. The laser beams, which carry 65 megabytes of information per second, have to be accurate within a range of 200 metres over a distance of 45,000 km.

A visit to the Zeiss-Jena research laboratories shows a company trying furiously to pull itself up by its bootstraps. A satellite dish antenna has replaced the red star atop the corporate headquarters in central Jena, but the workers know that competing in the West will take much more than that. "We had to develop from scratch technologies that already existed in the West because we weren't allowed to buy them," says Klaus Mütze, former director of research at Zeiss-Jena. "A lot of our scientists, especially the older ones, are having trouble making the transition."

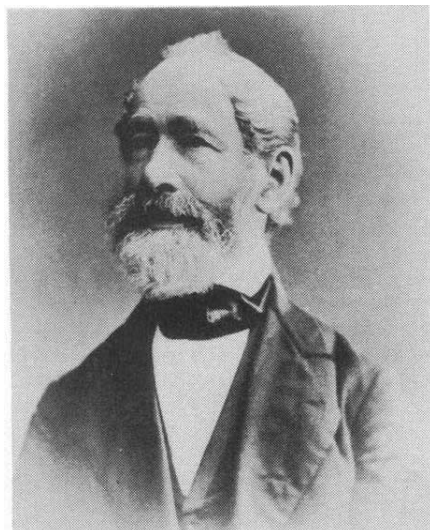
No one doubts, however, that parts of the company are worth preserving. "There are brilliant people at Zeiss-Jena who have rightfully retained the hard-earned German

RESEARCH FUNDING

reputation for precise work," says one Western researcher, expressing a commonly held view. If Zeiss-Jena were to go under, he adds, it would be "the end of an era".

Outside analysts say that the worst can indeed be avoided. One analyst from the US consulting firm SRI International, which is developing a concept for the reconstruction of the optoelectronics industry in the Jena region, says that the ability to do such high-technology projects as the hexapod telescope distinguishes Zeiss-Jena from eastern German companies in other fields. This may help buy time for the company, he says. Assuming — and this is the key assumption — that sufficient financial support and retraining of workers is provided over the next five years or so, the analyst says he sees an "excellent chance" for Zeiss-Jena to survive, either as one company or a number of smaller ones.

Zeiss-Jena managers say their strategy includes plans to develop or expand in new



Carl Zeiss, founder of the optics company, pictured in about 1885.

areas, such as lasers for medical and measuring applications, optical coatings and devices and digital image-processing, in addition to trying to expand sales to the West in Zeiss's traditional strengths — predominantly microscopes and measurement technology.

Whatever happens, Zeiss-Oberkochen is likely to play a major role in its long-lost sibling's future. The company was very annoyed at being shut out of the original talks between Thuringia and the trust body, and now it says that, if necessary, it is ready to go to battle over Zeiss-Jena. If the company is given to Thuringia, then Zeiss-Oberkochen may well go to court to determine who has the rights to the Zeiss name, says Manfred Berger, a spokesman for the western German company. "But we're still assuming we'll find a mutually acceptable solution."

Until then, Western researchers and Zeiss employees will be watching closely to see if capitalism can do in less than two years something that even 45 years of Communism could not achieve: destroy the historic Carl Zeiss-Jena.

Steven Dickman

Tough times in South Africa

Johannesburg

THE South African research councils and the universities are faced with their toughest financial constraints ever, following the budget recently delivered to parliament by Minister of Finance Barend du Plessis. The budget was characterized by large increases in spending on police (to attempt to curb civil unrest) and foreign affairs (to establish new missions abroad in the wake of the thaw in South Africa's foreign relations), but little or nothing more for research.

Despite an increase in government spending of 14 per cent (roughly in line with inflation), four of the five research councils suffered cuts in their parliamentary grants. The 1991–92 allocations to the councils were as follows (with percentage increase or decrease in brackets):

- Council for Industrial and Scientific Research (CSIR), R207m (£44.23m) (–2.4%);
- Foundation for Research Development (FRD), R109m (£23.29m) (–4.8%);
- Human Sciences Research Council (HSRC), R61m (£13.03m) (–1.9%);
- Council for Mineral Technology (Mintek), R52m (£11.11m) (+2.7%);
- Medical Research Council (MRC), R39m (£8.33m) (–5.0%).

The variation in allocations can be explained by the fact that the councils are funded through different government departments, which fared differently at the

hands of the Treasury. The cuts inflicted on the CSIR and MRC were lower than those in their funding departments' budgets: Trade and Industry and Health received cuts of 25.3 per cent and 12.1 per cent respectively. Mintek received its increase despite a 5.8 per cent cut in the budget of the Department of Mineral and Energy Affairs. The remaining councils, the FRD and the HSRC, along with the universities and national museums, are funded through the Department of National Education, which received a 1.6 per cent increase in its budget. The universities received exactly the same subsidies as last year, but at least negotiated their way out of a cut (see *Nature* 348, 183; 1990). The national museums, including the largest natural history museum, have also suffered severe cuts for the first time.

The CSIR, which is responsible for technologically orientated research conducted by its own laboratories, raised 46 per cent of its budget last year by contract work done for the private sector and government departments. According to its president, Dr Brian Clark, it hopes to increase this figure to 51 per cent this year, providing for an overall planned growth in income of 8 per cent. The FRD, MRC and HSRC are responsible for the provision of studentships, as well as the funding of research at universities, technikons and museums, so this sector is set to suffer most.

Michael Cherry

ANIMAL RESEARCH

Challenge on testing

Washington

As the battle lines of the animal-rights struggle shift, animal toxicity tests such as the LD-50 and Draize procedures have become the subject of challenges across the United States and in Congress. Last month, both the California and Vermont state assemblies passed bills outlawing skin-irritation and ocular (including Draize) testing on animals in the state. The bills will now be voted on in their respective state Senates. Since 1987, the number of states that have considered such legislation had tripled, to nine in 1990. Six are already debating animal-testing bills this year, and more are expected before the end of the year.

In the US Congress, Senators Harry Reid (Democrat, Nevada) and Barbara Boxer (Democrat, California) intend to introduce bills to restrict or outlaw animal testing for cosmetics and household products. Although both have tried and failed to pass such legislation in the past, research advocates consider the new bills to be a serious threat. "The trend is obvious," says Barbara Rich, executive vice-president of the National Association for Biomedical Research. "Toxicity testing will be the big issue this session". Christopher Anderson

The end for monkeys

Washington

GOVERNMENT scientists experimented on and killed two of the last four remaining "Silver Spring monkeys" last week after a last-minute legal battle that reached the US Supreme Court.

Officials at the National Institutes of Health (NIH) said the animals were dying of complications from previous injuries. Rescued by activists in 1981 from a laboratory in Silver Spring, Maryland, the monkeys have been a *cause célèbre* for the animal rights movement and the symbolic poster children of antivivisectionism.

Last week, the activist group People for the Ethical Treatment of Animals appealed to the Supreme Court after failing in lower courts to gain a restraining order so that the poor health of the animals could be confirmed by an independent veterinarian. Although the high court temporarily halted the experiment for one day, it permitted the killing of the monkeys after NIH said the animals were in pain, had ceased eating and would soon die. NIH researchers placed the animals under "terminal anesthesia" before examining their brains for signs of neural reorganization as an adaptation to injuries suffered in the original Silver Spring experiment.

C.A.

No growth for British science

SIR — Terence Kealey's rosy-hued vision of the growth of British science (*Nature* 350, 370; 1991) defies belief. I must draw attention to the gross and chronic inadequacy of the public monies available through the so-called dual-support system for funding the indirect (but real) cost of research whose direct costs are provided by research councils and UK charities. The facts are as follows.

(1) The Department of Education and Science (DES), the Universities Funding Council (UFC, formerly the University Grants Committee, UGC) and the Committee of Vice-Chancellors and Principals (CVCP) have all urged universities to recover the full costs of all research undertaken, rather than drive themselves into insolvency. In particular, we were urged to adopt the Hanham formula which shows that the typical level of indirect cost is 60 per cent of the direct costs for any given project.

(2) For research where the direct costs have been borne by research councils and UK charities, the indirect costs, under the dual-support system, have been provided as DR by the UGC/UFC. (DR is UFC research money distributed to universities according to their research grant income from the research councils and charities.)

(3) However, compared with the 60 per cent required, DR has never exceeded 31.5 per cent and has declined steadily to 23 per cent. This comparison between 60 per cent and 23 per cent is unaffected by international comparisons, gross domestic product, inflation or anything else. It represents chronic failure of the dual-support system to do what it is supposed to do. In 1990–91, the shortfall across the system is £160 million.

(4) Recent case analyses conducted by the research councils and several universities (including University College London) confirm the 'Hanham 60 per cent' and will no doubt influence the sum sought by the Advisory Board for the Research Councils from the UFC to meet the proposed transfer of responsibility.

(5) The shortfall, though, is not a result of that transfer, but is, rather, the result of gross underfunding of the UFC by the DES and the Treasury.

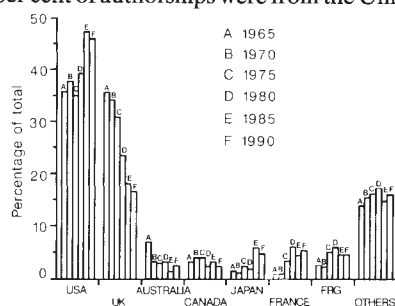
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SIR — We have carried out a survey of the contribution of laboratories from various countries to publications in *Nature* over the past 25 years. Between seven and ten issues of *Nature* were taken randomly for each year examined, and studies were carried out at five-yearly intervals from 1965 to 1990. For Articles and Letters, the number of publications in which a particular country was represented in terms of authorship was recorded. Thus, papers involving authorship

from two or more laboratories in the same country scored only one for that country, and authorship from more than one country scored one for each country.

The figure shows the trends in relative representation of different countries to authorship of *Nature* Articles and Letters since 1965. A very clear decline is seen in the United Kingdom contribution: in 1965, 34.6 per cent of authorships were from the United



Vitamins and IQ

SIR — There has been a lively discussion in *Nature* (350, 2 & 5; 1991) of a report attributed to "Californian research" that a vitamin—mineral supplement will increase IQ in children. The editor (page 3) speculates that the effect would put schoolteachers out of work, but the need for them is increased by eager and more intelligent pupils.

Why did the story produce a complete lack of interest in California? One possible explanation is that Californians consume so many vitamins that they are too intelligent to believe it. At the same time, we feel a certain unkind satisfaction that the United Kingdom, where criticisms of Californians' exuberance are complacently aired, should have been so easily hoodwinked. Peter Aldhous (page 5) calls for peer reviews. Perhaps the real problem is one of gullibility.

One obvious question was not raised in *Nature*. The product was a vitamin—mineral supplement containing ten minerals. Why is the alleged effect attributed to vitamins rather than to minerals? What about signal transduction by calcium?

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Patent confusion

SIR — F. W. Cousins' comments (*Nature* 340, 184; 1991) on the "withdrawn" British patent applications for zidovudine (formerly known as AZT) indicate a lack of knowledge of the current patent system. It is fairly common practice to establish a formal priority date by filing a basic application in the United Kingdom, then on foreign filing 12 months later to file an application in Europe designating the countries in which cover is

Kingdom, whereas the value fell to 16.7 per cent in 1990. This fall in UK contribution was compensated for by a rise in US authorships (34.6 per cent in 1965 to 45.8 per cent in 1990) as well as by a rise in authorship representation from other countries, in particular Japan, France and Germany.

Assuming that *Nature* accepts articles solely on the basis of scientific merit, we suggest that these data provide clear evidence for a decline in international competitiveness of British scientific research. With the continuing fall in government support for basic research in the United Kingdom, it is pertinent to ask whether this decline will continue.

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desired and claiming priority from the British application. That itself is generally not pursued any further by the applicant, in due course is "deemed abandoned" by the British Patent Office and five years after first filing the Patent Office file is destroyed.

In the case of zidovudine, this has now occurred with basic British applications which were filed in 1985 and 1986 and to which Cousins referred. The European patent, which Cousins does not cite, is 000196185.

The German Offenlegungsschrift 3608606 was similarly abandoned, its claims also being covered by the European patent.

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Too many noughts

SIR — I would like to call your attention to an error in "Bush asks for 13 per cent extra for science" (*Nature* 349, 443; 1990). You state that the Human Genome Project is in line for a \$334 million increase. This is clearly an editorial slip, because that sum far exceeds the combined genome budgets of both the National Institutes of Health (NIH) and the Department of Energy (DOE). As your table shows, the increase requested for the combined NIH and DOE genome programmes is in fact \$35 million. Of this NIH have requested an increase of \$23 million.

The uninformed reader could get a false and exaggerated impression about the size of the US budget for genome research from your article, creating unnecessary concern.

JAMES D. WATSON

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Science and secrecy

Walter Ormerod

Centrifugation techniques developed for uranium enrichment have been widely available for commercial exploitation and military use, but biologists have not been permitted to use them.

THE House of Commons inquiry into the American blacklist of 31 firms alleged to have been front companies for Iraq will need to be concerned with past as well as recent history. Certainly firms, such as the Technology Development Group, an Iraqi owned company, and other organizations with foreign allegiance have been able to export, from countries such as Britain, information and hardware, including designs for centrifuges for the enrichment of uranium. But this concern is somewhat belated; the decisions from which this situation has arisen were made many years ago. The following account of events some 25 years ago suggests, obliquely, how this situation may have arisen from the inability of the government to choose between the priorities of science, military secrecy and commercial exploitation.

In 1961, I was working on a project involving the separation of cell particles from *Trypanosoma brucei*, the agent of African sleeping sickness. I had the good fortune to employ a technician, Jim Molloy, who in an amateur capacity had become fascinated with model building and in doing so had become an accomplished engineer. He came to work with me when he was 23, having left school at 18 with a single 'A' level in chemistry: he was employed as a junior hospital laboratory technician when, after two years, we learned that his technique of 'zonal density-gradient centrifugation' was being developed by Norman Anderson at the Oak Ridge National Laboratory (ORNL) in Tennessee. I obtained a grant of £300 from the Wellcome Trust for Jim to visit ORNL; he spent two weeks there in May 1964.

Zonal density-gradient centrifugation entails the introduction of a solution of increasing concentration into a rotor spinning at a speed that is low yet sufficient to maintain the gradient. A suspension of particles is then introduced and the rotor spun at the correct speed so that bands representing the different particle sizes form (see Fig. 1). The rotor is then brought back to the loading-unloading speed, the gradient pumped out and the individual bands separately collected.

Three rotors

Anderson had been developing four types of zonal rotor: the A rotor, which had over a litre capacity and developing a force of 500g, was used for separating cells or larger organelles; the B rotor, developing 5,000g, was designed for the separation of particles of the general size of viruses and ribosomes; the C and D rotors, developing 300,000g and

1.2×10^6 g respectively, were designed for the separation of protein and nucleic-acid molecules. The A rotor was planar and the separations could be observed through a transparent cover, but the others were generally cylindrical and enclosed, allowing higher speed and greater safety.

Jim was given access to the design and operation of A and B rotors and was told how he could get access to the basic design of the C rotor, whose bearing was based on the design of the Zippé gas centrifuge. ORNL semiannual reports gave details of the C rotor, but we obtained a copy of the original report by Gerlot Zippé from the library of the UK Atomic Energy Authority (AEA) at Capenhurst. Jim made a model of the pivot bearing which is the basis of the gas centrifuge.

In the next few months, he was able to make a working version of the A rotor together with a machine for generating the necessary gradient solution. The rotor was a dangerous piece of equipment protected only with sand bags and I have a vivid memory of Anderson, visiting my laboratory, ducking behind a bench as the rotor passed through a 'critical', a period of instability of the accelerating rotor. At the same time Spinco, subsequently a division of the Beckman Corporation, brought out a commercial

B rotor at a cost of \$16,000, a sum greater than the total annual budget for my trypanosome work.

Because the rotor work was clearly beyond the scope of my original research proposals, I approached the UK Medical Research Council (MRC) for a grant of £5,000 for materials and equipment, but was told that the application would have to be "considered formally by the council". Because such consideration of a previous application had taken two years, I decided to accept an immediate grant of £2,000 from The Distillers Company Limited (DCL) which, because it included help from their workshops at Epsom, enabled Jim to complete the miniature version of the A rotor which we needed for our immediate objective of separating cell particles of trypanosomes (Figs 1 and 2). The centrifuge manufacturer MSE Limited (now a part of the Fison Group) helped us with the gradient engine and then marketed a version based on Jim's design.

The A rotor had a limited application mainly because of its size, whereas our miniature version was designed for use with smaller quantities of material. The B rotor is now an important part of biologists' equipment and is marketed by Beckman and others.

While Jim was in Oak Ridge, he attended an 'industrial cooperation conference on

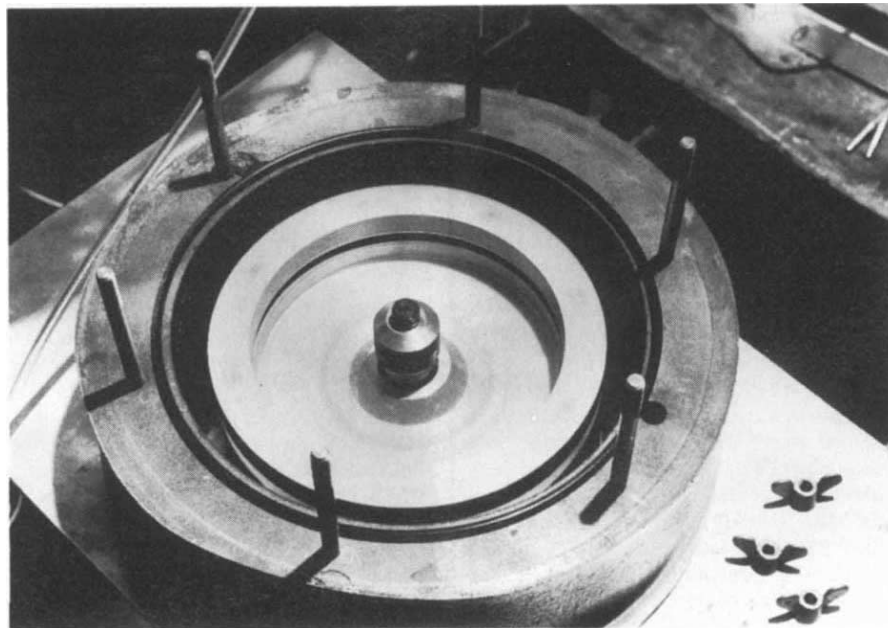


FIG. 1 Miniature rotor based on the ORNL A rotor constructed by J. O. Molloy shown in rotation. The protective sheath is a section of a gun barrel; the protective cover of 5-cm perspex has been removed. Two bands consisting of cell granules from the cytoplasm of *Trypanosoma brucei* can be seen.

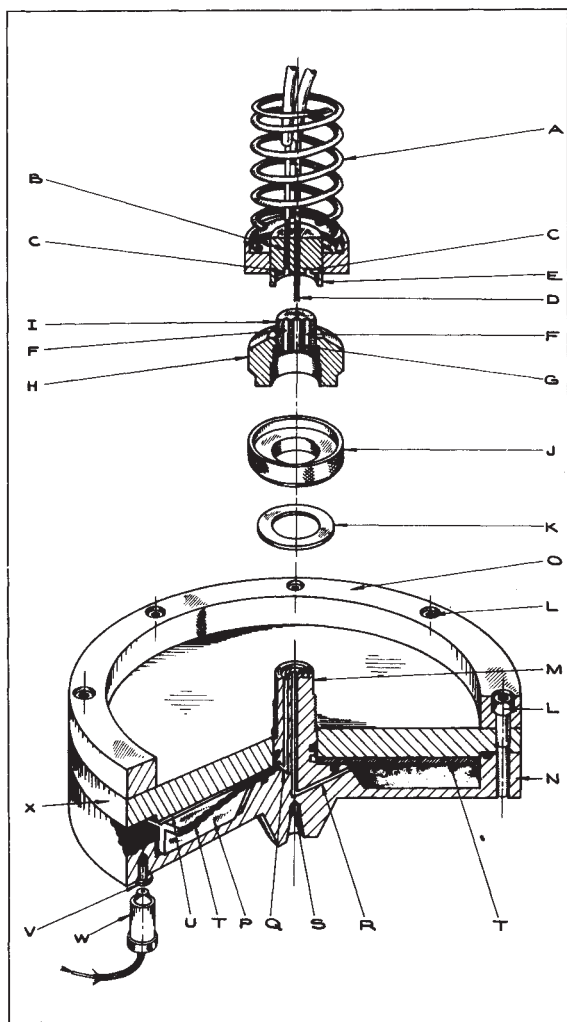


FIG. 2 Exploded view of the rotor shown in Fig. 1 (the width of the rotor itself is 15 cm). A–E, removable seal; A, spring; B, lateral feed tube; C, slotted groove; D, central feed tube; E, bush; F, peripheral drill holes; G, central drill hole; H, bush; I, rotating Rulon seal; J, nut; K, washer; L, screws; M and N, rotor body; O, alloy ring; P, septa; Q, core outer drill holes; R, inner drill holes; S, left-hand thread; T, perspex disk; U, groove in disk; V, activator screws; W, magnetic pickup; X, perspex lid. (From ref. 1.)

zonal centrifugation'. Also attending the conference was W. G. Marley, a British scientist from the AEA at Harwell. Marley had become aware that Jim was interested in the C rotor, which (at least in Britain) was classified as secret on the grounds that it was based on the design of the centrifuge being developed for uranium enrichment. Shortly after Jim's return, Marley paid us a visit; he shared our belief that the C rotor had important biological potential, he was also concerned that the secret status of the rotor should not be put in jeopardy. Marley therefore called a meeting on 11 August 1964, at the MRC's offices under the chairmanship of Sir William (the late Lord) Penney, chairman of the AEA. Jim and I were both present at the meeting.

Nobody at the meeting could suggest an application of the C rotor to the projects on which they were involved, although J. C. (now Sir John) Kendrew, of the MRC Laboratory of Molecular Biology, pointed

out that the potential of a C rotor could only be assessed if it became available. So Jim and I were the only group with any real interest in the C rotor: we were, indeed, becoming more interested in its development than in its use which, as the meeting had shown, was more speculative than essential. Nevertheless, we made it known that we wished to develop a C rotor, aided if possible by MSE and/or the DCL.

Although unstated, it became clear that our commercial link was unacceptable to the AEA. This was confirmed when I was invited to a meeting at Capenhurst, where the centrifuge operations of the AEA were based. At the meeting, no longer under the benign influence of Marley, we were supposedly to discuss technical aspects of the construction of zonal centrifuges, but I was not permitted to take Jim, who was now considered a security risk on the grounds that he had been involved in design and construction of zonal rotors with a company that specialized in making centrifuges.

The upshot of the Capenhurst meeting was that I was to abandon any proposal to develop a C rotor, and that the AEA would itself construct one and make it available to those who wished to use it: the apparatus was to be located at the then closed laboratories of the Government Microbiological Establishment at Porton Down. But this seemingly generous offer meant my complete withdrawal from the

project, partly because it would have meant beginning the project again with unfamiliar personnel and inadequate funds, partly because of the difficulties of working with infective material in both London and Porton, but particularly because I knew that Jim would continue to work with me, on the derisory salary offered to a technician, only if he had a chance of building a C-rotor centrifuge.

The security services made it clear that I had no option in the matter. They pointed out that the technique of gas centrifugation was being so closely guarded because it was the basis for a method of enriching uranium which was cheaper and easier than then current gaseous diffusion techniques, and because it could possibly be used by other countries for the development of a nuclear bomb.

So I abandoned the project, the Porton Centrifuge Facility came to nothing and Jim left to become a sales representative at a

higher salary and with a tenured position. Like any other citizen, I felt that I had paid a small price for limiting the spread of nuclear weapons.

That remained the position for the next four years, until it was announced that an intergovernment consortium consisting of Britain, Germany and the Netherlands had been set up for the production and marketing of enriched uranium obtained by gas centrifugation. The secrecy which had prevented my research project had been for commercial, not for military, purposes. Obviously I felt cheated; the short time interval seemed to indicate that the decision to widen the availability of the gas centrifuge as a commercial source of enriched uranium had already been made at the time that Jim and I were prevented from developing a C rotor for biological purposes.

There was little general protest at this development, although Leonard Beaton writing in an article in the *Times* newspaper entitled 'Sales before Sanity' (27 May 1970) noted that the consortium was tendering for the construction of gas-centrifuge plants in other countries. Thus, gradually, gas-centrifuge techniques for uranium enrichment have become widely known and now Iraq has them. Libya, Pakistan, Argentina and others are probably also in the know.

Lessons

There are still two important lessons to be drawn from this cautionary tale of 25 years ago: first, excessive secrecy defeats its own end. In the United States, nuclear research is often carried out by commercial firms under contract; ORNL, for instance, was at that time operated by the Nuclear Division of the Union Carbide Corporation. In Britain, nuclear research is carried out in government institutes whereas commercial firms and universities tend to be kept at arms length. Such exclusions highlight the 'secretness' of a project and thus enhance its desirability to a potentially hostile foreign power.

Second, important decisions about the exploitation of scientific advances should not be made solely by government departments working in secret. Government servants can easily misunderstand the scope of their decisions when there is none of the accountability that should be inherent in a 'freedom of information act', such as that of the United States. The decision to restrict information on the gas centrifuge to an international commercial operation was probably made by a small caucus without consideration of its consequences. The decision may yet prove calamitous. The UK Official Secrets Act seems to have been a barrier only to the legitimate use of information but not against potentially hostile military use. □

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Secret Service as ultimate referee

Statistical tests applied to Dr Thereza Imanishi-Kari's data are a vivid measure of the intrusiveness of external investigations and a proof of the need for caution in making use of material such as this.

HAS the US Secret Service become every journal's referee of the last resort? And what will be the consequences for ordinary mortals? Those are questions provoked by the report two weeks ago by the US National Institutes of Health's Office of Scientific Integrity (OSI) on the Imanishi-Kari case (see *Nature* 350, 262; 28 March 1991) which relies largely for its allegation that Imanishi-Kari fabricated data on forensic investigations in the past few years.

OSI's report is chiefly concerned with the notebook submitted under subpoena by Imanishi-Kari and David Baltimore to the Dingell Committee in 1988, two years after the publication of the disputed article in *Cell*. The Secret Service, commissioned by the committee and OSI, claims to have 6,000 samples of ink in its possession, and was on no-doubt familiar ground in punching 1-mm holes in pieces of printed paper to tell (by chromatography) how the ink concerned was constituted. What it found was that the notebook incorporated a mosaic of pieces of printed paper tape from a γ -counting machine which, although offered as a record of the disputed experiments, had nothing to do with them.

So far, nobody has dissented from the conclusion that many of the pieces of tape date back to at least 1984, long before the disputed work was carried out in 1985. (Some crucial data were gathered, or were said to have been gathered, in June 1986.) OSI's inference is that either the experiments described in the 1986 article were never carried out or that, if they were, the data "must have been deemed" unreliable or otherwise unsatisfactory. Even for the sake of completeness, it does not allow the possibility that the data were gathered, recorded — and then lost, which is uncharitable. But Dr Margot O'Toole's complaint, in 1986, was, among other things, that the disputed data did not even then exist.

But OSI and the Secret Service have also ventured into what must be less familiar territory with their analysis of the statistical properties of the rounding errors and background counts generated by γ counters, as recorded in the Dingell notebook. These are the investigations on which two members of OSI's scientific advisory panel, Drs Ursula Storb and Hugh O. McDevitt, issued a separate and sceptical opinion, noting that "this type of analysis is new and untried".

Much of the argument is about rounding errors. The Dingell notebook, now supposed to be an after-the-event composite, contains strings of handwritten numbers supposedly

derived from measured γ -counts by rounding. In that trade, consecutive samples, including "blanks" or controls, are carried beneath γ -counters. Recorded measurements may be numbers of the order of 10,000 in "counts per minute" for live samples and fewer than 1,000 for the controls. It seems to be common practice to round recorded measurements. A count of 567 measured may, for example, be converted to 570 when transcribed to a notebook.

The Secret Service has probably now produced the most comprehensive analysis ever of people's behaviour in the reduction of the significance of numbers by rounding them. Its assumption is that people inventing numbers do not behave as if they were random-number generators, but rather make choices from a restricted set of numbers.

Its most striking observation is that, in one set of 265 handwritten data recorded in the Dingell notebook (pages 124 to 128A), a set of numbers each smaller than 600, there is a marked tendency for numbers rounded to the tens digit to end in 1, 3, 7 and 8. Indeed, 205 of these 265 data have these digits in their least significant places, while the values 0 and 5 are underrepresented, at least if the underlying distribution of the tens digit were uniform. Using chi-squared analysis, OSI concludes that the chance of this pattern arising by chance is less than 1 in 100,000.

For what it is worth, these data are said to have been derived from experiments carried out in June 1986 (after the publication of the original paper, but also after it had been challenged), and were pleaded in aid in the correction published in *Cell* two years later. To be fair, the Secret Service has also worked through data in other people's notebooks as well as through uncontroversial data from the Dingell notebook itself to show that departures from expectation on such a scale do not ordinarily crop up.

But are there circumstances when departure from the simple uniform distribution might be expected? Inevitably, there are. A person transcribing data from a printed tape to a notebook page might, for example, reasonably slip into a frame of mind in which some of the digits between 0 and 9 seemed more appropriate than others.

Suppose, for example, that one has it in mind that the inaccuracy of a measurement is 10 per cent, and one has a series of measurement between 200 and 500 in which even the last digit is nominally significant. The objective of rounding is to get rid of spurious accuracy, but even the standard rounding rules will not accomplish that. If for example, 543

is represented after rounding by 540, That leaves an embedded expected error of 54, but it is not possible to say in which direction.

Even so, why not go a step further by rounding towards a smaller set of digits in the range from 0 to 9, say the even digits (including zero) or perhaps the set 3, 6 and 9? The answer, of course, is that anything is allowable, but that even rounding-rules should be made explicit. And the snag apparent in the Dingell notebook is that any such rule in Imanishi-Kari's head was evidently not followed consistently; least significant digits from all over the range crop up, but with too low a frequency.

OSI offers still further elaborations. For example, in the analysis of sets of γ -counting records, it tests the possibility that a γ count may reflect the behaviour of a finite number of radioactive signals, each represented by a different Poisson distribution. This is evidently a way of generating records with a degree of spikiness, but not so much that the more deeply suspect data can be accounted for. This part of OSI's argument would be more convincing if more were known of the origins of the background counts which biologists — and some cold fusioners — seem content to accept uncritically.

A further test to which the Imanishi-Kari data have been put concerns the measurements of radioactivity in a set of wells in a plastic plate in which cell fusions took place and whose occurrence should be strictly a matter of chance. Somebody had the idea of correlating a sequence of measurements with the same sequence displaced by a fixed number. What emerges is that there is a periodicity in the data (with a tendency to repetition at displacement intervals of 12 and 24 that would not be expected of an underlying randomness (and which is primarily used to argue that the data concerned represent quite different measurements)).

Storb and McDevitt are right to say that the use of methods such as this is so unfamiliar that few will base serious conclusions on them. If the Dingell notebook had not already been shown to be a composite, it is unthinkable that they would have been persuasive in the Imanishi-Kari case. Even here, their most useful function is to show inhomogeneity in apparently homogenous sets of data. It remains to be seen which journals test sets of data submitted for publication for spikiness as defined by the Secret Service, but authors bent on fabrication (none of them readers of this journal) will no doubt in future ensure access to a random-number generator just in case.

John Maddox

An agent of suppression

Winship Herr

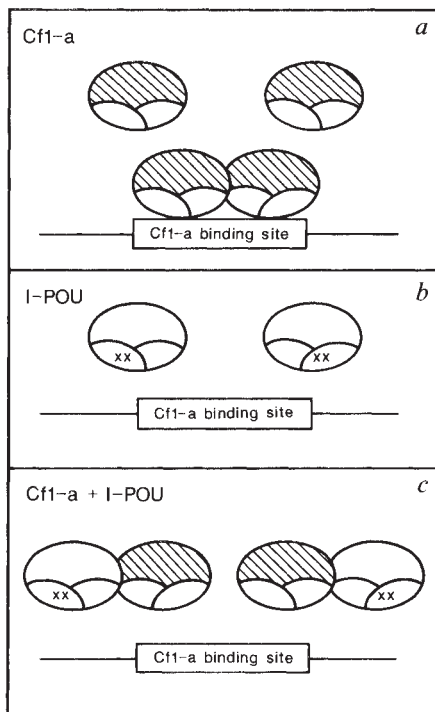
IN *Drosophila*, homeodomain proteins determine patterns of development by regulating transcription through sequence-specific interactions with DNA. Frequently, however, the DNA-binding specificity of homeodomain proteins observed *in vitro* does not explain their regulatory activities *in vivo*. In human cells and yeast additional regulatory specificity can be acquired through protein-protein interactions, but hitherto no such examples have existed in *Drosophila*. This has now changed with the discovery by Treacy, He and Rosenfeld, reported on page 577 of this issue¹, of an unusual interaction between two *Drosophila* homeodomain proteins that results in inhibition of DNA binding and suppression of transcription. Both proteins are POU-domain proteins except that one of them, called I-POU for inhibitory POU, does not bind DNA; instead, through the formation of very stable heterodimers, it can prevent the second POU protein, Cf1-a, from binding to its own regulatory targets.

The POU proteins are a unique subfamily of homeodomain proteins that are distinguished by a bipartite DNA-binding domain, of 150–160 amino acids, called the POU-domain^{2,3}. The homeodomain (60 amino acids) lies in the C-terminal portion of the POU-domain; the N-terminal region contains a 75-amino-acid motif particular to the POU proteins called the POU-specific region. Linking these two conserved motifs is a hypervariable segment of 15–27 amino acids. Although the POU homeodomain can bind DNA weakly on its own, the entire POU domain is required for high-affinity and sequence-specific DNA binding^{3–6}. POU proteins display the interesting ability to bind DNA either as monomers or cooperatively as dimers. For example, the mammalian B-cell POU protein Oct-2 activates transcription through the octamer motif ATGCAAAT which it recognizes as a monomer⁷ and yet it can also bind cooperatively to appropriately juxtaposed octamer motifs⁸.

Treacy *et al.* discovered the I-POU protein in a search for POU-domain sequences in *Drosophila*. The POU-domain of I-POU is very closely related to the POU-domain of unc-86, a POU protein that regulates neuroblast differentiation in *Caenorhabditis elegans*, but it differs from unc-86 in one curious respect — I-POU is missing two of five basic residues that are present in the N-terminal portion of all other POU-homeodomains. In the homeodomains of the Antennapedia and engrailed proteins this basic region reaches into and contacts bases in the minor groove^{9,10}. Consistent with the critical importance of these residues for DNA binding, I-POU does not measurably bind DNA, but its binding

activity can be activated by inserting the two missing basic residues into the I-POU homeodomain.

If I-POU cannot bind to DNA, what is its function? It turns out that, by forming specific and very stable heterodimers with the Cf1-a protein, it can prevent Cf1-a from binding to its regulatory target in the dopa decarboxylase (*Ddc*) gene and thus inhibit transcription of this gene¹. Cf1-a



Schematic representation of the interactions between Cf1-a, I-POU and DNA. *a*, Cf1-a (hatched objects) is monomeric in solution but dimeric when bound to the Cf1-a regulatory site in the *Ddc* gene. *b*, I-POU which lacks two basic residues (xx) that are critical for DNA binding, is a monomer in solution and unable to bind to DNA. *c*, Coexpression of Cf1-a and I-POU leads to stoichiometric heterodimer formation between Cf1-a and I-POU. These stable heterodimers no longer recognize the *Ddc* regulatory site.

was first discovered in the process of cloning genes that encode regulators of a critical *cis*-acting element that activates expression of the *Ddc* gene in selected dopaminergic neurons of the fly¹¹. The cell-specific pattern of Cf1-a expression is complicated, involving epidermal tissues and neuronal cell lineages, but is consistent with a role of Cf1-a in regulation of the *Ddc* gene¹. In contrast, expression of I-POU is restricted to a specific subset of neurons. In keeping with there being a functional interaction between Cf1-a and I-POU, these two proteins are coexpressed in a highly overlapping subset of cells in the nervous system.

The figure shows the regulatory interactions between Cf1-a, I-POU and DNA that Treacy *et al.* have uncovered. As illustrated in *a*, in the absence of its binding site Cf1-a is primarily monomeric but cooperates to form homodimers when bound to its regulatory target in the *Ddc* gene, whereupon it induces *Ddc* transcription. As shown in *b*, I-POU is also apparently a monomer in solution, but unlike Cf1-a it displays no ability to recognize DNA. Together Cf1-a and I-POU form very stable heterodimeric complexes at a stoichiometry of 1:1, and (see *c* in the figure) the heterodimer does not recognize the Cf1-a binding site nor could the authors detect binding of the heterodimer to a random set of DNA sequences. Although in Cf1-a the POU-domain is sufficient for association with I-POU, the regions within I-POU required for association with Cf1-a were not determined. Should it turn out that only the unusual I-POU homeodomain is required to bind to Cf1-a, this result may portend the existence of I-POU equivalents ('I-HOMEO') among non-POU homeodomain proteins.

The I-POU-Cf1-a complex is more stable than the Cf1-a-DNA complex, and therefore addition of I-POU to DNA-bound Cf1-a rapidly leads to dissociation of Cf1-a from its binding site¹. But, as the authors point out, coexpression of I-POU with Cf1-a is not necessarily incompatible with transcriptional activation by Cf1-a, because the interaction between the two proteins is stoichiometric and therefore overexpression of Cf1-a can overcome the inhibitory effects of I-POU. In this light, I-POU may serve as a buffer to inhibit the activity of Cf1-a when expressed at low levels in inappropriate cell types. So the synthesis of Cf1-a need not be shut off tightly in cells where its activity would be deleterious.

The inhibitory effect of the basic-region deletion in I-POU on DNA-binding by Cf1-a is reminiscent of the inhibitory effects that the helix-loop-helix (HLH) protein Id, which also lacks basic residues essential for binding to DNA, has on DNA-binding by MyoD and other HLH proteins¹². There could be one significant difference, however. Whereas HLH proteins are never known to bind to DNA as monomers, POU-homeodomain proteins are able to bind and to activate transcription through high-affinity

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monomer binding sites, such as the highly conserved octamer motif in the case of the proteins Oct-1 and Oct-2. Thus it is possible that in some cases I-POU or related proteins may suppress transcription directed by regulatory sites that require dimer formation for effective binding, and yet not adversely affect activation of transcription directed by

CARCINOGENESIS

Testing times for the tests

Alastair Hay

WITH the appearance of the latest issue of *Mutation Research*, further fat is added to the fire of controversy over assays for potential cancer-causing agents. R. W. Tennant and J. Ashby^{1,2} find that a crucial distinction in such tests should be made between agents that damage DNA and those that do not, and that *in vitro* testing for the second group is severely flawed.

These conclusions run counter to one of the themes of a series of papers published last year by Bruce Ames and colleagues³⁻⁶. Ames is the originator of the Ames test for identifying mutagens, and hence potential carcinogens, using *Salmonella*. In the papers he and his co-authors questioned established procedures for testing carcinogens in animals and also challenged the wisdom of concentrating so much regulatory attention on synthetic pesticides when there are far more natural pesticides in plants, some of which are undoubtedly carcinogens. The outburst was a potent blend of common sense and speculation, calculated to ruffle feathers. It was not a challenge that could simply be brushed aside, for Ames is no Don Quixote and the targets of his attack are far from imaginary. The message, however, has still to be taken on trust. This did not prevent (among others) the UK Ministry of Agriculture Fisheries and Food, which was under attack from consumer groups for its apparent lack of concern about synthetic pesticides in food, from using the 'natural pesticide' argument to justify its position on food residues.

At the centre of Ames's questioning, however, was the manner in which carcinogens are tested in animals. Regulators, quite rightly, place much more emphasis on a positive carcinogenicity test in animals than, say, a positive mutagenicity test in *Salmonella* or mouse lymphoma cells, or results from any other *in vitro* short-term test. Although rapid to carry out (they provide an answer in days rather than years), *in vitro* mutagenicity tests will not identify all carcinogens; they are more likely to pick out chemicals with reactive chemical groupings that can attack DNA (structurally alerting chemicals) and which cause cancer in several species and at several sites^{7,8}.

In one survey of 224 chemicals, the Ames test had a sensitivity (percentage of carcinogens identified as mutagens) of only 54% and a specificity (percentage of non-carcino-

monomer binding sites. If true, this would serve as a unique example of how protein associations can lead to restriction of transcription factor activity to a subset of its recognition sites. □

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gens identified as non-mutagens) of 70% (ref. 9). And in a survey of 73 carcinogens and non-carcinogens, the *Salmonella* assay only identified some 45% of carcinogens¹⁰. So if Ames is right about the way that rodent bioassays are used to test for carcinogenicity, these performance figures are actually better than they seem. He believes that the dosing schedules used in rodent bioassays lead to many chemicals being falsely identified as carcinogens, some positives being simply an artefact of sustained tissue injury⁴.

The highest of the two dose levels that are usually used in bioassays is referred to as the maximum tolerated dose. This is the highest dose that can be given without the animal showing any gross signs of toxicity, and not more than about 10% weight loss. Maximum tolerated doses are invariably higher — sometimes an order of magnitude more — than the doses to which humans are likely to be exposed. Because they are so high, Ames believes that the chemical concentration is

Salmonella mutagenicity data, divided into structurally alerting and non-alerting agents, and by carcinogenic status

Carcinogenic status	Salm. positive (no. in group)	
	Structurally alerting	Structurally non-alerting
All chemicals	78(154)	4(147)
All carcinogens	84(105)	4 (57)
Two-species carcinogens	93 (58)	5 (22)
Single-species carcinogens	72 (47)	3 (35)
Equivocal evidence	69 (16)	4 (23)
Non-carcinogens	66 (33)	5 (67)

From ref. 2.

sufficient to kill cells. The damage is compensated for by new cells being synthesized, and it is this increased cell proliferation (mitogenesis) which is the root cause of the problem. For as Ames points out, "a dividing cell is much more at risk for mutation than a quiescent cell"⁴, with DNA (single-stranded rather than double-stranded during cell division) being more vulnerable to damage, and the available time for DNA repair being much shorter when a cell is dividing. Cell division will also increase the expression of certain oncogenes.

Increased cell turnover plays a part in the development of tumours but only a part, for carcinogenesis is regarded as a multi-stage process that probably involves mutations at

numerous genes. Ames is now saying that cell division is the crucial factor. His critics point to the fact that doses sometimes well below the maximum tolerated dose are also used in animal studies, and that these often cause cancer but not in so many animals. This dose-response effect (the higher the dose the greater the effect) is regarded by most toxicologists as providing more evidence of a cause-and-effect relationship.

But the acid test of Ames's thesis will be monitoring cell turnover *in vivo*, and this is difficult to carry out for a large number of carcinogens and non-carcinogens. For the moment, such studies as have been done to see whether organs damaged by chemicals also become cancerous have not been convincing. The US National Institute of Environmental Health Sciences recently reviewed 53 chemicals that are animal carcinogens. Of these only seven showed a clear link between organ toxicity and tumour development. For the other 46, cancers occurred in organs with no apparent damage, and visibly damaged organs had no tumours¹¹.

A more pragmatic approach to identifying carcinogens is the use of appropriate short-term tests and animal bioassays. A combination of the *in vitro Salmonella* assay and an *in vivo* test such as the mouse bone marrow micronucleus assay will identify the vast majority of established human carcinogens¹². In their reports in *Mutation Research*^{1,2}, Ashby and Tennant use a slightly different approach to judge whether a chemical might cause cancer in humans. By comparing the mutagenicity to *Salmonella*, the structural

features of a chemical which determines whether it would react with DNA, and the carcinogenicity in rats and mice of 301 chemicals, they dissect out the genotoxic (DNA-damaging) from the non-genotoxic carcinogens and non-carcinogens. The information on the 301 chemicals comes from the National Toxicology Program database,

the carcinogenicity data being summarized in a series of tables. Ashby and Tennant show that there is a high correlation between structurally alerting features and mutagenicity, but that the correlation of either property with carcinogenicity is low (see table). In other words, neither property is sufficient to identify rodent carcinogens.

To unravel any other messages in the data, the authors sub-divided their 154 structurally alerting chemicals and 147 non-alerting chemicals into six broad chemical groups. From this subdivision it is apparent that most rodent carcinogens, and certainly most of those which cause cancer in both the rat and mouse and/or at several sites, are among the structurally alerting chemicals. Most structu-

rally alerting chemicals are mutagenic — 84% of the carcinogens and 66% of the non-carcinogens. All of the 33 aromatic amino/nitro type, two-species carcinogens are mutagenic. For structurally alerting chemicals the *Salmonella* assay showed high sensitivity (88%) but low specificity (33%). Of the 147 non-alerting chemicals — of which 57 are animal carcinogens — only 6 (fewer than 5%) were mutagenic.

Ashby and Tennant conclude that it is pointless to discuss the sensitivity and specificity of the *Salmonella* assay without defining the broad group to which a chemical belongs. The chemical class will determine the result rather than the assay itself. If, for example, an environment were to be screened for potential carcinogens, the *Salmonella* assay will detect the vast majority of the aromatic amino/nitro groups, but will not identify chlorinated aromatics.

The way that the information is set out will provide other researchers with a database that can be interrogated intelligently. Thus, the position of a chlorine atom may be crucial. Neither 1,2-dichlorobenzene nor 1,4-dichlorobenzene are mutagens, yet 1,4-dichlorobenzene causes kidney tumours in the rat and liver cancer in the mouse. Additional chlorine atoms also affect the outcome of carcinogenicity testing. For instance 2,4-dichlorophenol and 2,4,6-trichlorophenol have no structurally alerting features and are not mutagens, yet the second of them causes haematopoietic cancers in the rat and liver tumours in the mouse.

In the final analysis it is the underlying mechanism of carcinogenesis in animals that is important, and whether it is relevant to humans. Aromatic nitro/amino groups on chemicals are structurally alerting features and many chemicals with these features are mutagens in *Salmonella*. Yet, in terms of their carcinogenicity, this 'family' is as disparate as all the others, ranging from potent, two-species, multisite carcinogens such as the dye CI Basic Red 9, to the non-carcinogen 4-nitro-*o*-phenylenediamine, a chemical with three alerting structures attached to a benzene ring. Nevertheless, the fact that DNA damage is probably the stimulus for carcinogenicity with such compounds indicates that a possible hazard to human health exists. But the principle may not apply

to the non-genotoxic carcinogens, hence the importance of Ashby and Tennant's claim that the genotoxic-non-genotoxic divide is an appropriate classification for carcinogens.

In support, the authors point out that some markers are uniquely associated with genotoxicity, for example tumours of the lung and Zymbal's gland in rodents. Four other tissues seem to be involved in non-genotoxic carcinogenesis and the underlying mechanisms are worthy of exploration, they claim. Chemicals that cause renal tumours in the rat kidney (such as 1,4-dichlorobenzene) by increasing kidney tubular concentrations of the protein α_2 microglobulin and disrupting calcium reabsorption¹³, or increase peroxisome proliferation in rodent liver, or which cause leukaemia or thyroid tumours in rodents, may do so by disturbing the normal genetic control in these sensitive tissues, rather than by the chemical specifically damaging DNA. Thus, say Ashby and Ten-

nant², non-genotoxic carcinogenicity is more subtle than the "simple concept of tissue toxicity" put forward by Ames — chemical features can be used to identify potentially genotoxic carcinogens, but homeostatic disturbances induced by other chemicals in individual tissues, rather than direct damage to DNA, determines non-genotoxic carcinogenicity. So use of specific *in vitro* and *in vivo* mutagenicity tests will only identify genotoxic carcinogens.

Three years ago the same authors performed a similar exercise on 222 chemicals⁷. They have now gone further and identified crucial areas of research which will improve our knowledge of mechanisms of rodent carcinogenicity, and the relationship between carcinogenicity in animals and that in humans. □

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A way-out 'asteroid'

ON 18 February, Rob McNaught of the University of Adelaide discovered a fast-moving asteroid in the south-polar skies. Earth-approaching asteroids are frequently found these days, but later observations soon revealed that McNaught's object (provisionally designated 1991 DA by the International Astronomical Union) has an orbit around the Sun that is exceptional for an asteroid.

Although 1991 DA was observed while between the orbits of Earth and Mars, its trajectory shows that every 41 years the 5-km diameter object goes out beyond the orbit of the planet Uranus. No other asteroid ventures so far from the Sun. The much larger, enigmatic object Chiron, which was originally designated an asteroid, has in recent years been found to show cometary emissions; even it stays at least 3 astronomical units closer to the Sun (1 AU is the distance between the Sun and the Earth) than 1991 DA.

The orbit of 1991 DA is also strongly tilted (62°) to the plane of planetary orbits in the Solar System: it looks like the orbit of a comet. Several astronomers have observed 1991 DA closely, but there is no hint of a coma, the glowing gaseous head that is always evident around comets so

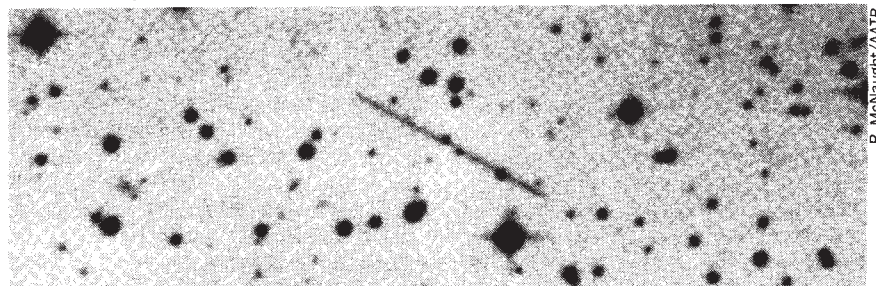
close to the hot Sun.

Comets that become trapped inside Jupiter's orbit usually lose their volatile ices and 'die' by the time they have made several hundred passages through the inner Solar System. Evidently, longer-period comets normally get dislodged from orbits like that of 1991 DA long before they can become devolatilized. When they venture close to Jupiter's strong gravity field, they either become trapped in shorter orbits (later to die) or else are ejected far beyond Jupiter.

Conceivably 1991 DA is an asteroid that has somehow recently been dislodged from the asteroid belt. It is much more likely that it is really a comet that has been lucky enough (perhaps because of its high orbital tilt) to avoid close encounters with massive Jupiter, Saturn or Uranus. Survival for tens or hundreds of thousands of years might be long enough for it to lose its volatiles during its brief visits to the inner Solar System. If so, this 'asteroid' is actually a rare burned-out comet.

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The asteroid (comet?) 1991 DA was first identified from the streak traced across this plate recorded with the UK Schmidt Telescope in Australia.

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All together now . . .

Ian Stewart

ONE of the most spectacular displays in the whole of nature occurs in South-East Asia, where huge swarms of fireflies flash in synchrony. As H. M. Smith (*Science* **82**, 151; 1935) described the phenomenon:

Imagine a tree thirty-five to forty feet high, apparently with a firefly on every leaf, and all the fireflies flashing in perfect unison at the rate of about three times in two seconds, the tree being in complete darkness between flashes. Imagine a tenth of a mile of river front with an unbroken line of mangrove trees with fireflies on every leaf flashing in synchronism, the insects on the trees at the ends of the line acting in perfect unison with those between. Then, if one's imagination is sufficiently vivid, he may form some conception of this amazing spectacle.

Why do the flashes synchronize? Renato Mirollo and Steven Strogatz (*SIAM Journal*

that Mirollo and Strogatz have proved.

Peskin's mathematical model involves a large number of identical oscillators, with fully symmetric coupling — that is, such that each oscillator affects all of the others in exactly the same manner. The component oscillators are of the 'integrate-and-fire' type, in which a voltage-like variable increases up to a threshold value, and as soon as it reaches this threshold the oscillator 'fires' and the variable drops back to zero.

The most unusual feature of Peskin's model is that the oscillators are pulse-coupled. That is, an oscillator affects its neighbours only at the instant when it fires; and it then increases the appropriate variable in each neighbouring oscillator by a fixed amount, or causes it to fire if the variable would exceed the threshold. The mathematical difficulty is to disentangle all of these interactions. Mirollo and Strogatz analyse a class of models similar to, but more general than, the one proposed by Peskin. They prove that, no matter what the initial conditions are, eventually all of the oscillators become synchronized. The proof is based on the idea of absorption, which happens when two oscillators with different phases 'lock together' and thereafter stay in phase with each other. Because the coupling is fully

symmetric, once a group of oscillators has locked together, it cannot 'unlock'. The idea is that a sequence of absorptions eventually locks all of the oscillators together, and a geometric and analytic argument is used to show that this "almost always" happens.

The authors remark that many other systems of biological oscillators tend to function in synchrony: "the pacemaker cells of the heart, networks of neurons in the circadian pacemaker and hippocampus, the insulin-secreting cells of the pancreas, crickets that chirp in unison, and groups of women whose menstrual periods become mutually synchronized". It now seems that all of these systems attain synchrony by the same mathematical mechanism: absorption in networks of pulse-coupled oscillators.

They also formulate a conjecture of their own: that synchrony is also the rule in any connected network of pulse-coupled oscillators, and not just in networks with fully symmetric coupling. In other words, the fireflies will synchronize even when some of them can't see some of the others, provided no isolated group exists that cannot see any of the rest. But in these more general networks absorptions can be 'unlocked' by subsequent interaction, so a new method of proof will be needed. Mathematicians remain in the dark about these more subtle aspects of firefly synchronization. □

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IMAGE
UNAVAILABLE
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REASONS

Firefly flashing — an "amazing spectacle".

of *Applied Mathematics* **50**, 1645–1662; 1990) have (the cliché is irresistible) shed a great deal of light on this problem by proving a mathematical conjecture due to C. S. Peskin. They show that synchrony is the rule for mathematical models in which every firefly interacts with every other.

It is natural to model the insects as a population of oscillators, coupled together by visual signals. In most previous work on the synchronization problem, the coupling between these oscillators is assumed to be smooth. But, in 1975, Peskin formulated a mathematical model in which the oscillators are coupled discontinuously (*Mathematical Aspects of Heart Physiology*; Courant Institute of Mathematical Sciences, New York). He conjectured that in this model, for almost all initial conditions, eventually all oscillators become synchronized. It is this conjecture

Sooty superconductors

Arthur W. Sleight

BUCKMINSTERFULLERENE, the soccerball-shaped allotrope of carbon, continues to yield surprises at a breathless pace. Barely weeks after it was revealed that doping films of C_{60} with potassium renders them conducting, we now learn on page 600 of this issue¹ that cooling the doped material to 18 K makes it superconducting. This remarkable, unanticipated finding can be regarded as forging a link between organic and inorganic superconductors.

Although the molecular structures of the principal fullerenes, C_{60} and C_{70} , are now well established, the structures of the solids obtained on condensing these molecules are only poorly known. The C_{60} films which were doped with potassium to become conducting and superconducting were amorphous².

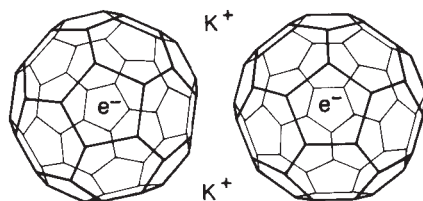


FIG. 1 Interstitial potassium atoms could give up an electron to the C_{60} molecules.

Nevertheless, well crystallized C_{60} and C_{70} have been prepared. In crystals, the pseudo-spherical molecules tend to take on either a cubic close-packed or hexagonal close-packed arrangement³. In the case of pure C_{60} , the cubic arrangement tends to be preferred. At room temperature, these pseudospheres rotate about their centres. Thus, although there is a periodic array of pseudospheres, there is not a well defined periodic array of carbon atoms. On cooling to 249 K, there is a first-order phase transition where at least much of this rotation apparently ceases⁴.

Like graphite, C_{60} is expected to be a reasonable electron acceptor, and a charge transfer salt containing the C_{60}^- anion has been prepared⁵ by electrolysis: $(Ph_4As^+C_{60}^-)(Ph_4As^+Cl^-)$. A team at AT&T Bell Laboratories also recently found² that when C_{60} is exposed to alkali-metal vapours, it becomes electrically conducting. Now this group has found superconductivity in several samples of K_3C_{60} . The actual composition of the superconductor is unknown but may be close to K_3C_{60} . The detailed structure of this phase is also unknown, but there is adequate space for potassium cations to be placed in-

In step

AMONG the first minerals to crystallize from basaltic magma are chromites and olivines, which may therefore be expected to record the magma's initial chemical composition. No so, warn P. A. H. Scowen *et al.* (*Contrib. Mineral. Petrol.* **107**, 8–20; 1991) — studies of chromites from Kilauea Iki lava lake in Hawaii show that they remain in equilibrium with the evolving lava composition, even when entirely encased within olivine. The lava lake was created in 1959 during an eruption of Kilauea and its rapidly changing composition has been closely monitored since. The fact that the chromites' composition also changed in so short a time indicates that they must exchange ions with the lava quickly, even when not in direct contact with it.

More from less

It may not satisfy the homeopaths, who claim the power to detect substances that are completely absent, but P.G. Gillespie and A.J. Hudspeth (*Proc. natn. Acad. Sci. U.S.A.* **88**, 2563–2567; 1991) have devised a technique for the recognition of femtogram quantities of proteins, minor constituents for example in a single cell. The total protein in a mixture — in one case the contents of a retinal rod outer segment — is covalently labelled with biotin, before or after gel electrophoresis. After blotting (with certain precautions that go beyond the use of coffee whitener to block non-specific binding) it is detected on film by a chemiluminescent reaction, catalysed by avidin-coupled alkaline phosphatase. Use of a similarly conjugated second antibody is another option. A range of applications suggests itself, as for instance the detection of foreign proteins expressed in *Xenopus* eggs.

No two ways

Two tribes of experimenters in the physics of fundamental forces could be unwitting antagonists, suggest S. K. Lamoreaux *et al.* (*Europhys. Lett.* **14**, 503–505; 1991). Workers trying to find out if neutrons can turn freely into their antiparticles hope to constrain attempts to develop 'grand unified theories' of the strong and electroweak forces of matter, whereas those trying to find out whether gravity acts in different ways on matter and antimatter hope to learn something of the quantum description of gravity. In their note, which brings together authors from each camp, Lamoreaux *et al.* point out that any difference in the gravitational interactions of neutrons and antineutrons would give them different energy making their interconversion impossible to observe. But the observation of neutron–antineutron conversion would greatly limit the possible difference in their gravitational energy.



FIG. 2 The apparatus of Hebard *et al.* The superconducting doped C_{60} film is on the glass square near the bottom. The sealed tube contains inert helium to protect the fragile film.

terstitially between close-packed C_{60} spheres (Fig. 1). We can be relatively certain that the potassium has not found its way inside the pseudospheres. The potassium-doped C_{60} materials are highly reactive: the powders are pyrophoric, and the conducting films rapidly lose their conductivity on exposure to air. Greater stability might be possible if appropriate dopants could be placed inside the pseudospheres.

Another allotrope of carbon, graphite, can also be doped with potassium, through an intercalation reaction. In this case, however, the material is metallic both before and after intercalation. Intercalation of potassium into graphite also produces superconductivity⁶, but only at temperatures below 1 K. In graphite, there is extended covalent bonding in two dimensions with weak Van der Waals bonding between the layers. For C_{60} , there is covalent bonding within the pseudospheres but only weak Van der Waals bonding between pseudospheres. On doping C_{60} , we may assume, potassium atoms lose an electron to the pseudospheres, partially populating a conduction band throughout the solid derived mainly from the $2p$ orbitals of carbon but with some contribution from the $2s$ orbitals. These conduction electrons may well tend to bond the pseudospheres together, thus aiding in the electron transfer from pseudosphere to pseudosphere. There is every reason to believe that potassium-doped C_{60} is isotropic. Some have felt that the two-dimensional character of the cuprate superconductors is a key factor for their high

transition temperature. However, at least for carbon, three-dimensional bonding is apparently better for superconductivity than is two-dimensional bonding.

Both C_{60} and C_{70} are properly regarded as new allotropes of carbon. However, they differ from the more familiar diamond and graphite allotropes in an important way. Both diamond and graphite are forms of carbon that are thermodynamically stable as solids over certain ranges of pressure and temperature. Molecules of C_{60} and C_{70} might well exist at thermodynamic equilibrium at very high temperatures in gas-phase carbon, but the solid forms of C_{60} and C_{70} are unlikely to be thermodynamically stable at any temperature or pressure. The point is important, because superconductivity is often associated with instabilities. Consistent with this association is the finding of 'high-temperature' superconductivity in the allotrope of carbon which is never stable as a solid. The

copper and bismuth oxides that exhibit high-temperature superconductivity also appear to be metastable, at least at the temperatures at which they are superconducting⁷. Thus, ways of preparing new superconductors often involve tricks to defeat equilibrium. This can lead to challenging synthetic chemistry, as with C_{60} and K_3C_{60} .

Throughout the short history of superconductors, it was generally believed that highest transition temperatures would be found for transition-metal compounds. The discovery of superconductivity in perovskite oxides of bismuth^{8,9} gave the first exception. $(Ba,K)BiO_3$ now yields a transition temperature¹⁰ of 34 K, and superconductivity persists up to 18 K in K_2C_{60} . Superconductivity is known for compounds based on the intermediate rows of post-transition metals, but the transition temperatures do not exceed 7 K. Surely, there are more superconductors to be discovered in this region of the periodic table. □

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ASTRONOMY

X-rays from γ -ray bursts

Charles D. Dermer

OVER 500 γ -ray bursts have been detected through satellite surveillance of the sky in the two decades since they were first discovered. These cosmic transients flare up, emit photons with energies comparable to the electron rest-mass energy, and fade from γ -ray sight within a few seconds to a few tens of seconds. Except for three sources emitting anomalously short and low-energy spectra, no γ -ray burster has been seen to repeat, nor has there been any confirmed association of bursters with steady sources radiating longer-wavelength photons. This has led astronomers to mount simultaneous observing programmes to monitor bursts at X-ray and optical wavelengths, with the goal of detecting either direct emission at lower energies, or reprocessed, fluorescent or scattered burst radiation. X-ray afterglows, which may be the decaying embers of the burst source, have been observed, and were in fact predicted by some models. Now Murakami and co-workers, on page 592 of this issue, supply another piece of the puzzle. Using data from the X-ray detector on the Ginga satellite, they confirm that X-ray emission may significantly precede the γ -ray photons, a fact which both supports and confounds current neutron-star models for γ -ray bursts.

The nature of γ -ray bursters remains unknown. Unlike the radio pulsars and X-ray bursters which are found mostly in the plane of our Galaxy, γ -ray burst events are isotropically distributed on the celestial sphere. Statistical tests of the size distribution of bursts have also failed to establish a distance scale, and therefore an energy budget, of the

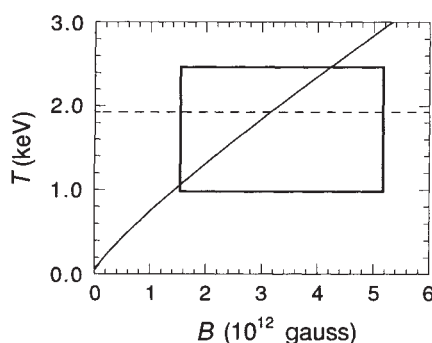


FIG. 1 Eddington temperature of a 1.4-solar-mass neutron star radiating thermal emission. The gravitation force balances the Compton-scattering force on an electron-proton plasma above the neutron at the temperature given by the solid line, which depends on magnetic field B as shown. The Eddington temperature for a nonmagnetized neutron star corresponds to about 2 keV, as shown by the dashed line. The box indicates the range of temperatures and magnetic fields deduced from γ -ray burst observations. (Although not shown here, the line pressure from magnetic Compton scattering becomes weaker than the nonmagnetic Compton pressure at $B \leq 10^9$ gauss.)

bursters. This has led to imaginative schemes for the origin of the bursts, including models involving cosmic strings or colliding neutron stars outside our Galaxy.

Up against the evidence of the statistics, which lends no support to the hypothesis that γ -ray bursts originate in our Galaxy, is the circumstantial evidence of the spectra. Features at energies between 20 and 80 keV are thought to result from cyclotron scattering of photons by plasma electrons embedded a 10^{12} -gauss magnetic field; and features near 400 keV are generally interpreted as 511-keV radiation from electron-positron annihilation that has been redshifted to lower energies by the intense gravitational field of a collapsed star.

The evidence implicates collapsed stars with strong magnetic fields — that is, neutron stars. But these arguments are not compelling: line features are observed in fewer than one in five bursts, and simultaneous confirmation is lacking. Moreover, the strong magnetic fields invoked seem to be at odds with observations of photons with energies in excess of a megaelectronvolt: these would materialize into electron-positron pairs as they crossed the field.

X-ray observations of γ -ray bursts test the neutron-star hypothesis in several ways. As shown by Murakami, Yoshida and their co-workers, the spectra of the early and late X-ray phases resemble those from a hot object. From the spectrum one can calculate the body's temperature, which in turn indicates the energy flux from the object, so that it is a simple matter to relate the size of the radiating region and the distance to the source. The Ginga X-ray fluxes are in accord with emitting areas of around 1 km² and distances about 1 kiloparsec (the galactic disk in our vicinity is about 0.3 kpc deep). Neutron stars are thought to have radii of about 10 km, so 1 km² represents a reasonable size for an X-ray hot spot. A 1-kpc scale height for neutron stars might, however, seem rather large. But radio pulsars, which could well be the progenitors of γ -ray bursters, are born with velocities of 100 km s⁻¹ or more, and are launched to heights of several kiloparsecs above the galactic disk.

Another indirect piece of evidence comes from the 'Eddington' limit: if the luminosity of a compact source is too large, matter is blown out by the force of radiation pressure. This limit on luminosity is a benchmark for black-hole studies of quasars and active galactic nuclei, where the fuel is thought to come from the gravitational accretion of matter. An unusual twist occurs in a highly magnetized object such as a neutron star. The Compton-scattering of X- and γ -rays by electrons is modified by the restriction of electrons to discrete orbits called Landau levels, so that cyclotron-line absorption is

the main source of radiation pressure, much as atomic-line absorption generates the pressure driving stellar wind.

Thus accreting plasma, or material on the surface of the neutron star, can be blown away if there are enough photons emitted at the electron cyclotron energy. This energy is equal to $11.6 B_{12}$ keV, where B_{12} is the magnetic field in units of 10^{12} gauss. Figure 1 shows that a magnetic field on a neutron star

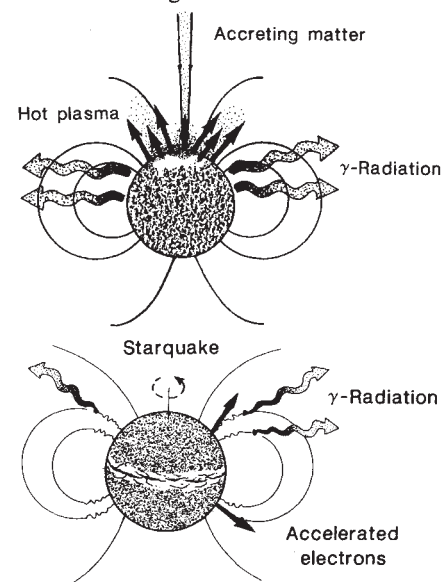


FIG. 2 Schematic diagram illustrating two popular models of γ -ray bursts. In the thermonuclear model (top), the energy source results from nuclear burning of material accreted either transiently or steadily. In the starquake model (bottom), the energy source derives from the gravitational or rotational energy in the neutron star, and radiated through Compton scattering by relativistic electrons. (Figures by K. Hurley.)

radiating as a blackbody can have the effect of either reducing or increasing the Eddington limit relative to the nonmagnetic value. For a neutron star with $B_{12} \approx 2-5$, implied by the cyclotron lines in γ -ray burst spectra, the transition between sub-Eddington and super-Eddington conditions corresponds to temperatures between about 1 and 2.5 keV ($10-30 \times 10^6$ K), precisely the range of temperatures derived from the Ginga data.

It is tantalizing to speculate that super-Eddington heating of the neutron star surface could trigger a γ -ray burst. This heating could be accomplished through accretion, as in the thermonuclear-fusion model, or by the release of the neutron star's rotational or gravitational energy, as in the starquake model (Fig. 2). X-ray emission is certainly expected from thermonuclear burning, but not in advance of the burst. Even if X-rays help to trigger the burst, the problem here is to propose an efficient mechanism to channel fusion energy into γ -rays, which carry more than 90 per cent of the burst energy. A starquake could heat the neutron star's surface, and provide photons that could be Compton-scattered by a current of rela-

tivistic electrons in the magnetosphere, much like a radio pulsar. How relativistic nonthermal electrons are accelerated by a process making thermal X-rays remains unexplained. Still, the X-ray spectral signatures suggest that neutron stars are the sources of γ -ray bursts, and the results of Murakami *et al.* show that a vanguard of apparently thermal X-rays, which is clearly connected to the main portion of the burst,

ZOOLOGY

A whale of a new species

Katherine Ralls and Robert L. Brownell, Jr

ALTHOUGH four new species of whale have been identified since 1937, the absence of new ones for the past 28 years might be taken to mean that they had finally all been discovered. Not so — a new species of beaked whale, *Mesoplodon peruvianus*, is described by J. C. Reyes and colleagues in the latest issue of *Marine Mammal Science*¹.

The beaked whales, family Ziphiidae, comprise almost 30 per cent of the toothed whales but are poorly known. In contrast, the great baleen whales are well described, in part because they were commercially hunted, and no new species have been discovered since 1878, when the last of the ten now recognized, Bryde's whale, was named². Six of the seven beaked whales described in this century are in the genus *Mesoplodon* (see table), and most of what little is known about the genus, and the family as a whole, has been gleaned from carcasses washed ashore. One species (*M. pacificus*) is still known only from its bones and the

must be accommodated by a γ -ray burst model. Whether these objects are more closely related to radio pulsars or accreting neutron stars, or whether they turn out to be extragalactic flashers of a different order, has yet to be answered. □

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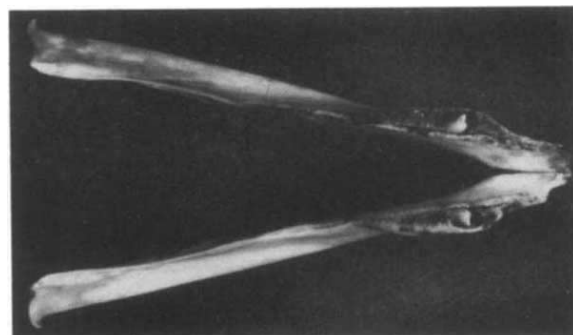
appearance of the whole animal remains a matter of conjecture.

Members of four of the five beaked whale genera have reduced numbers of teeth. The name *Mesoplodon* (loosely interpreted as "armed with a tooth in the middle of the jaw") refers to the two remaining functional teeth of adult males, which are on the lower jaw and are one of their most striking features. The bizarre teeth of the male strap-toothed whale (*M. layardii*) even grow up and over the upper jaw, sometimes actually touching and thus preventing their owner from opening his mouth more than about five cm (ref. 3). The amount of the tooth exposed in the living animal varies from species to species: in some nearly all the tooth is exposed, whereas in others most of it is covered by gum tissue and only the tip protrudes. Females also have the same two teeth in the lower jaw, but they are not functional as they rarely emerge from the gums. The meagre data available imply that these animals prey predominantly on squid and fish found at moderate to considerable depths³, a soft diet that can be captured and consumed without the aid of teeth.

In *Mesoplodon*, linear scars up to about 2 m in length are often found on adult males, and are usually assumed to be the result of intraspecific combat. These scars often occur as pairs of parallel lines, implying that they were formed by the two teeth of a rival male^{3,4}; males apparently keep the mouth closed while wounding other males. Members of the genus tend to occur as antitropical pairs of closely related species, a species of each pair occurring in the temperate waters of each of the two hemispheres, although one (*M. densirostris*) is found in tropical waters world-wide and two others (*M. ginkgodens* and *M. pacificus*) are confined to the warm waters of the Pacific and Indian Oceans.

The latest example of *Mesoplodon* was described from ten specimens collected from the central and southern Peruvian coast. Six,

and perhaps seven, of them were captured incidentally in drift gill-nets set for sharks, and three were washed ashore. Some new specimens from the Mexican coast may also belong to this species and its latitudinal range may extend beyond the places where both the known and the suspected specimens were collected. The teeth of the adult males are relatively small compared to those of its congeners. The long axis of each tooth is almost perpendicular to the long axis of the mandible (see figure), a feature which distinguishes this species from all others in the genus. *Mesoplodon peruvianus* is the smallest known member of the genus, and, indeed, of all the beaked whales, both in terms of total length at birth (159 cm) and the maximum known total adult body length of 372 cm. (The smallest previously known species of *Mesoplodon* is *M. hectori*, for which the smallest calf measured 190 cm in length and the maximum known adult total length is 443 cm.)



Dorsal view of the lower jaw of the holotype of *Mesoplodon peruvianus*¹. That only two teeth remain functional is characteristic of males of the genus; that their long axis is almost perpendicular to the jaw is characteristic of the species. Length of the jaw is about 53 cm.

There have been several sightings of a distinctive but unidentifiable species of beaked whale in the eastern tropical Pacific, meaning that there still may be at least one more undescribed species of *Mesoplodon*⁵. Given that Earth's dwindling large-mammal fauna is comparatively well catalogued, *Mesoplodon* is likely to remain the least known genus of large mammal — as Minasian and colleagues have pointed out in *The World's Whales*⁶, "The mysteries these strange whales present will not be easily solved by the handful of specimens chance provides in one lifetime". □

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Species of cetacean described since 1900 (refs 1,2)*

Andrews' beaked whale, <i>Mesoplodon bowdoni</i> Andrews, 1908
Spectacled porpoise, <i>Australophocaena dioptrica</i> (Lahille, 1912)
True's beaked whale, <i>M. mirus</i> True, 1913
Baiji (Chinese river dolphin), <i>Lipotes vexillifer</i> Miller, 1918
Longman's beaked whale, <i>M. pacificus</i> Longman, 1926
Tasman beaked whale, <i>Tasmacetus shepherdi</i> Oliver, 1937
Fraser's dolphin, <i>Lagenodelphis hosei</i> Fraser, 1956
Vaquita (Gulf of California harbour porpoise), <i>Phocoena sinus</i> Norris and McFarland, 1958
Ginkgo-toothed beaked whale, <i>M. ginkgodens</i> Nishiwaki and Kamiya, 1958
Hubbs' beaked whale, <i>M. carlhubbsi</i> Moore, 1963
<i>M. peruvianus</i> ¹ , Reyes <i>et al.</i> , 1991

*All 11 species are toothed whales, seven are beaked whales (family Ziphiidae), and six of these seven are in the genus *Mesoplodon*. The genus *Tasmacetus* also belongs to the beaked whales.

[†]As Reyes *et al.* provide no common name for the newly described species, we propose the name pygmy beaked whale.

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Two-layered awakening

R.S.K. Barnes

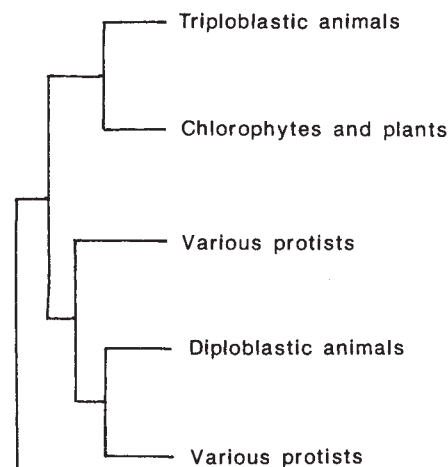
CLUE is a who-done-it with three different endings, all consistent with the evidence presented during the film. This scenario bears a remarkable resemblance to attempts to unravel the relationships of animals, on which new, molecular leads are reported by R. Christen and co-workers in *The EMBO Journal*¹.

There are (it is generally agreed) three basic animal body plans: the sponges with their two cell layers, cellular grade of organization and lack of a nervous system and symmetry; the radially symmetrical cnidarians, also basically with two layers of cells, but with a net of unsheathed nerve cells and tissue-level organization; and the bilaterally symmetrical others, with sheathed nerve cords, organ systems and, embryologically, three germ layers. But views on the interrelationships of the three types cover almost all possible combinations, from each being independently derived from the protists^{2,3} to all being descendants of an organism that was itself already an animal⁴, by way of any two groups (often the cnidarians and the bilateral phyla) being related to each other but not to the third line (in this case the sponges)⁵. Two enigmatic but simple smaller groups, placozoans and mesozoans, may then boost the number of times that animals have evolved from protists up to five (or more) or may be allied with one or other of the three major lines³. You pay your money and you take your choice.

Part of this regrettable state of affairs results from the lack of features shared by all animals but not with the heterotrophic flagellates from which they presumably sprang. All animals are, by definition, multicellular, but the dividing line between colonial protists and multicellular organisms is somewhat vague and many protist groups have colonial members whilst several include multicellular forms. Also, in all animal lines, at least ancestrally, adults are diploid and produce gametes by meiosis; but the same state occurs in some protists. The only feature unique to animals is that they develop from an embryonic blastula. Even here some protists have blastula form, although as adults, never as an embryo. Altogether this is hardly such a suite of uniquely shared characters as to inspire confidence that the animal condition could only have arisen once, particularly when multicellularity has arisen several times elsewhere.

Part of the problem is that there are no fossils that shed light on the interrelationships of early animals, and zoologists must therefore argue solely from the structure of surviving organisms. Hitherto most such argument has relied on gross or cytological anatomy, but Christen *et al.* have added fresh molecular evidence to the debate by sequencing part of the 28S ribosomal RNA molecule from three

sponges, three cnidarians, three ctenophores and a placozoan (groups which they unite as the 'diploblastic' animals and for which little molecular data is available), and then comparing their findings with earlier ones of members of the same team on the bilateral



The interrelationships of diplo- and triploblastic animals as deduced from 28S rRNA sequence data by Christen *et al.*¹. On this analysis, the triploblasts originated relatively early and are closer to the plants in ancestry than are the diploblasts.

animals (or as they term them, the 'triploblasts'), various protists, fungi and higher plants^{6,7}.

Several points emerge from their comparisons of nucleotide sequences. First, although plants as expected were closely associated with chlorophyte algae, no protists emerged as potential animal ancestors (although it is unfortunate that choanoflagellates were absent from their data because many would regard them as close to the origin of at least SEMICONDUCTORS

some animals⁸). Second, in the analysis diploblasts and triploblasts appeared to be well separated (see figure) with the triploblasts originating relatively early and being closer to the ancestry of plants than to that of the diploblasts. The sponges, cnidarians, ctenophores and Placozoa were clustered as a unitary group, all potentially descended from a single ancestor.

This raises a third point of interest. Classically, ctenophores were regarded as related to the cnidarians⁹, and were often united with them as 'coelenterates', although there have since been many suggestions of affinity with flatworms instead¹⁰. These sequence data place ctenophores firmly with the cnidarians and not with the bilateral flatworms. Finally, Christen *et al.* found no evidence linking ciliates with flatworms.

These analyses support notions that the animal condition has evolved more than once from a protist ancestry. They are unlikely, however, to be the last word on the subject, for not only are certain critical groups (such as the choanoflagellates) missing from the materials sequenced, but the information in 28S rRNA is only one of many disparate clues that have to be accommodated in this real-life detective story. □

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Dawn of the diamond chip

L. M. Brown

THE development, by a team at Tokai University, of an all-diamond p-n junction diode may be the crucial step towards realizing the dream of diamond-based semiconductor electronics. This would be equivalent to the electronics based on silicon and III-V compounds such as GaAs. Because of its electronic properties and strong bonding, diamond offers advantages in many applications. Diamond-based chips could operate at 1,000 °C, handle very high power, and operate in highly radioactive environments (for example, as radiation monitors inside nuclear reactors). And blue light-emitting diodes, not possible with conventional materials, could be made from diamond semiconductors. The new diode was made

possible by the development of a technique for doping diamond films with phosphorus to make the elusive n-type material.

Since the discovery of natural p-type semiconducting diamonds (the p indicates that they include elements that make one fewer bonds than carbon, making 'hole-type' conductivity possible), there has been much speculation that electronic devices might be constructed. Many devices based solely on p-type material have been made. These have largely relied on the 'Schottky barrier' formed when a metal such as aluminium is brought into contact with a semiconductor.

Although n-type diamond (containing five-valent elements that donate an extra electron to the diamond lattice) might be

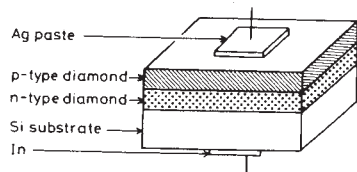


FIG. 1 Structure of the Tokai diamond diode (see text for details).

expected to result from nitrogen doping, and indeed most natural diamonds contain dissolved nitrogen, the extra electron on the nitrogen is too strongly bound and becomes ionized only at temperatures of several thousand degrees. Phosphorus has also been tried as an n-type dopant, ion implantation being used to insert it into the diamond. But the damage to the lattice caused by this technique is too great.

The Tokai work has been made possible by the advent of low-pressure techniques for making polycrystalline diamond films by chemical vapour deposition (CVD): n-type doping is then achieved by injecting diphosphorus pentoxide into the methane/hydrogen gas mixture used to form the diamond. The injection of boron trioxide allows the growth of p-type diamond, so that the same techniques can be used in diamond semiconductor fabrication as in conventional silicon technology.

The Tokai group now describe the first p-n diode junction produced in this way (K. Okano *et al. Solid St. Elec.* **34**, 139–141; 1991; *Appl. Phys. Lett.* **58**, 840–841; 1991). The diode was grown on a silicon substrate (Fig. 1) and the electrical connection was made using an indium/silver paste; melting of indium curtailed the range of operating temperatures. Furthermore the current rectification of the device (the purpose of a diode; Fig. 2) was rather poor at room temperature, owing to the high resistance of the n-type diamond. Nevertheless, after this first step, rapid progress towards an all-diamond transistor can be expected, and the question arises: what commercial applications might such devices have?

Although diamond conducts heat very efficiently, and so should make an effective heat sink, the high electrical resistivity of the doped films will impede operation at high power. Furthermore, the energy-levels of the donors and acceptors are still rather large, so the charge-carrier concentration in the diamond will be strongly temperature-dependent, unlike similarly doped silicon whose resistivity shows very little temperature

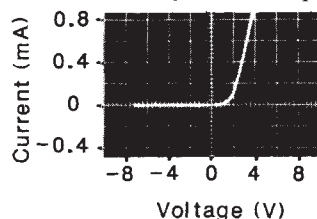


FIG. 2 Rectifying current-voltage characteristics of the Tokai diamond diode. (Both figures from *Appl. Phys. Lett.* **58**, 840–841; 1991.)

dependence over the operating range of the device. It is interesting to note that the threshold voltage for conduction in the pioneering devices reported by the group at Tokai seems to be just under 2 V, whereas with the dopants used one might expect nearly 5 V for perfect diamond, which has a very large gap between the conduction and valence states. Possibly the grain boundaries in the CVD films play an appreciable role in the conduction mechanism.

The use of diamond in light-emitting diodes may also suffer drawbacks: diamond has a so-called indirect optical band gap so that lattice vibrations must become involved for light to be radiated. The high energy of the vibrations of diamond's rigid lattice makes the process inefficient, so that useful light-emitting diodes may be difficult to achieve. The most promising line, for which there is considerable demand, may well be the production of robust radiation detectors. Already such devices exist, based on Schottky barriers, and according to some reports, the high-energy-physics Superconducting Super Collider will use diamond particle detectors. This ought to provide considerable development capital on about the right timescale.

Furthermore, the civilian nuclear power industry has an urgent need for in-reactor devices which can monitor conditions such as neutron flux, or mechanical movement, under intense radiation at high temperatures. Such devices might revolutionize reactor design, making it possible to detect the incipient development of dangerous conditions with much greater ease and certainty than is now possible. Silicon devices degrade so quickly as to be almost useless, but diamond-based devices should last at least an order of magnitude longer under reactor conditions.

The first practical requirement is to produce a sufficiently thick, reproducible, high-conductivity p-n junction to permit pulse counting of particle impacts, probably on an appropriate scintillator placed before or around the junction depending upon the nature and energy of the particles to be detected. The next requirement is to perfect a system of electrical contacts compatible with operation at high temperatures. One imagines that because of the uniquely valuable information obtainable from such detectors, the necessary capital and research capability may be forthcoming to make this application of diamond-based electronics a realistic possibility within the foreseeable future.

However, whatever the future holds, the remarkable achievement by the Tokai group of an all-diamond p-n junction is a milestone on the road to the production of an array of electronic devices based on a substance which in most people's minds is still just a girl's best friend. □

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Still sound

MANY rubbers and plastics can 'imbibe' liquid solvents, swelling up as they do so. Curiously enough, they can also imbibe high-pressure and supercritical gases. When the pressure is released, the imbibed gas sometimes bursts its way out of the rubber — hence the enigmatic destruction of many an apparently sound gas-seal. Daedalus now plans to exploit this effect.

Consider, he says, a rubber swollen by the absorption of high-pressure gas, and then restored to atmospheric pressure. The linked network of rubber molecules is under tension; without its loading of dissolved gas it would contract. The gas molecules, by contrast, are under pressure. Without the restraint of the rubber they would expand. At some well-chosen concentration of occluded gas, the two effects should balance exactly. The material would then have no preferred volume. Over a wide density-range, it could be squashed or expanded without developing any restoring force. It would be the first totally inelastic solid.

So DREADCO engineers are pressurizing various polymers in different gases, in search of this ultimately dead product. One problem is that it will be metastable. On removal from the pressure-chamber it will immediately start to lose gas by evaporation. The best compositions, however, should hold their gas almost immobile in the polymer lattice, rather like a conventional plasticizer. Even the small amount of gas which escapes from the surface will then simply leave a skin of dense, gas-depleted polymer to impede further evaporation. The interior of the product will have zero elasticity, and will therefore transmit sound with zero velocity. In other words, it will be a perfect sound insulator.

The modern world is crying out for it. DREADCO's "Dead Silence" polymer will be rapidly adopted in engine-covers for cars, lorries and motor mowers, decor for aircraft, mufflers for pneumatic drills, screens for offices, restaurants, and telephone booths, and shielding for industrial machinery. Householders in particular will welcome it. Nearly all the world's housing was put up before, or in total disregard of, the development of the amplifier. Now, at last, Dead Silence wallpaper will bring instant relief from the most rock-crazed neighbours. The modern town house, flat, or tower-block will become at least acoustically bearable.

Even the most stable Dead Silence formulations will slowly lose gas, and shift away from perfect density-equilibrium. They will then transmit sound, but at very low velocities. You will know that redecoration is needed when you start to hear through the wall the noises your neighbours made yesterday. David Jones

Stronger recycled plastics

SIR — Recycled plastics often possess relatively poor mechanical properties in comparison with products made from virgin materials. In particular, recycled plastic packaging materials are derived largely from polyolefins of low strength, weakened further by impurities in the recycled feedstock. Blending of a more rigid polymer with a polyolefin to form a stronger alloy is appealing economically but polymers commonly used by the packaging industry tend to be highly incompatible with each other^{1,2}. These problems notwithstanding, we have achieved good and consistent

mechanically tested. Both compressive³ and tensile⁴ properties were consistently either equal to or greater than values calculated by assuming a linearly weighted sum of contributions due to the pure components, for compositions up to 40 per cent PS. At 35 per cent PS, the stiffness (modulus) was optimal and anomalous, being 108 per cent (approximately 2 times) greater than that for pure PE.

Electron micrographs (see figure) of fracture surfaces reveal the size, shape and distribution (morphology) of each of the two components. For compositions up to 30 per cent PS, drawn fibres of PS are dispersed uni-

terized by more extensive regions of PS and thinner areas of PE. This dissolution of the uniformly entangled morphology is correlated with a decrease in the modulus.

We do not as yet fully understand why these blends have properties superior to those reported in previous studies, but this discovery strongly suggests that recycled blends made from incompatible polymer pairs may be formulated and processed in such a manner as to produce composite materials with good mechanical properties.

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Stretching a point

SIR — Those of us who do not work on the major histocompatibility complex (MHC) were very impressed to read in P. Parham's News and Views (*Nature* **348**, 674; 1990) that the MHC region of the genome is about 500 megabases in size. This would require about 17 per cent of the entire mammalian genome! Perhaps many of us have been unwittingly working on the MHC after all. Alternatively, is it possible that one or two extra zeros have crept in?

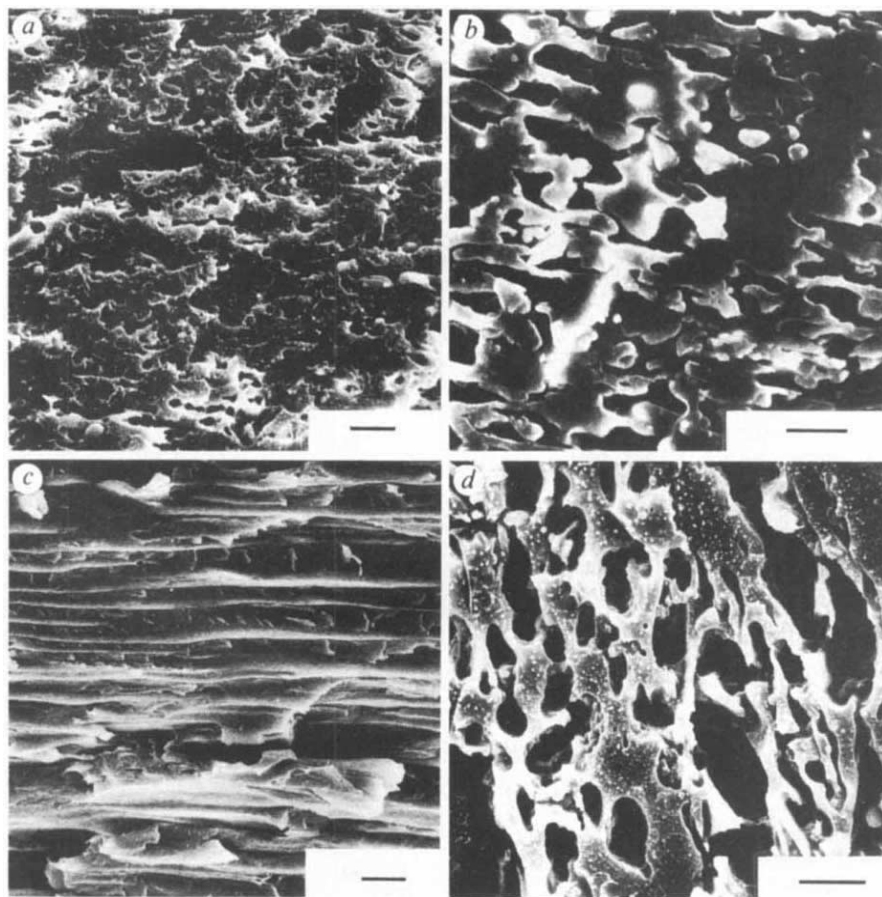
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[Somehow everything becomes bigger in California.
PETER PARHAM]

Scientific Correspondence

Scientific Correspondence is intended to provide a forum in which readers may raise points of a scientific character. They need not arise out of anything published in *Nature*. In any case, priority will be given to letters of fewer than 500 words and five references. □



Scanning electron micrographs of the surfaces of PS/PE blends. All samples shown were fractured at liquid-nitrogen temperature. Unless otherwise indicated, toluene was used to extract the PS phase, which appears as the darker of the two phases. *a*, 30/70 PS/PE. The surface is parallel to the length of the profile. Dark, circular/elliptical holes represent cross-sections of fibres/plates of PS in a matrix of PE. *b*, 35/65 PS/PE. Perpendicular view showing entangled phases. *c*, 35/65 PS/PE (no solvent extraction). The surface is parallel to the length of the profile. A laminate of rods and plates of PS and PE is evident. *d*, 40/60 PS/PE. Perpendicular view showing nonuniformity of the mixture and increase in extent of the PS phase. Scale bars, 10 μm.

mechanical properties in a blend of two of the most common packaging materials, polyethylene (PE) and a more rigid polymer, polystyrene (PS).

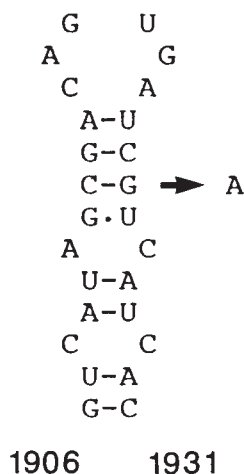
The PE component was derived from post-consumer plastic containers. Post-industrial PS scrap was mixed with the PE in various weight ratios. The molten mixtures were moulded into shapes with long, linear profiles of constant cross-section, and were

formly throughout a continuous matrix of PE; the morphology is similar to that of a fibre-reinforced composite, with the fibres down parallel to the length of the profile. At 35 per cent PS, the morphology is relatively uniform, with both polymers forming continuous phases that are entangled or interlocked. This morphology may be related to the enhanced strength at this composition. At 40 per cent PS, the morphology is charac-

Alzheimer's mutation

SIR — It has recently been shown¹ that affected individuals in two familial Alzheimer's disease pedigrees contain a base substitution in the gene encoding the amyloid beta protein precursor (APP) located on chromosome 21. This substitution replaces a G with an A at position 1924 of APP (according to APP695 sequence)², resulting in the production of an isoleucine in place of a valine at residue 642 in the transmembrane domain. The authors speculate that the slightly more hydrophobic isoleucine residue may alter the anchoring of APP in the membrane¹.

We offer an alternative hypothesis, and suggest that this mutation disrupts a regulatory stem loop structure in APP messenger RNA. Flanking the mutation site are 26 nucleotides capable of forming a stem loop structure (base pairs 1906–1931, see figure).



The stem loop formed by nucleotides 1906–1931 of APP (APP695 nomenclature). The CAGUGA loop matches a consensus sequence of iron-responsive elements, and so may be a site of translational regulation of APP. The substitution of G to A at position 1924 destabilizes the stem, disrupting this structure⁵.

This loop contains the consensus sequence CAGUGA characteristic of the iron-responsive elements (IRE) present in the genes encoding ferritin and the transferrin receptor³. These elements interact with a common binding protein which regulates protein production at the translational level in response to iron concentration. In both the ferritin and transferrin receptor genes, the IREs are contained in noncoding regions. In APP an IRE-like stem loop appears precisely at residue 40 of the beta/A4 peptide. By analogy, the putative IRE stem loop in APP may be a translational regulatory element which governs APP production.

The stem consists of ten bases on either side. Eight nucleotides on each arm form base pairs with the complementary side (including one G·U pair). The base substitution at position 1924 destroys the third of three consecutive base pairs directly under the loop, thereby destabilizing the stem (see figure).

Patients heterozygous for this mutation may lose the ability to regulate normally translation of APP and so produce abnormally high amounts of protein product. This would lead to elevated levels of APP in these patients similar to those observed in patients with Down syndrome who, presumably owing to the presence of a third copy of the gene, form substantial amounts of amyloid by middle age⁴.

Translational regulation of APP message by a protein similar to the IRE-binding protein would allow both environmental and genetic factors to influence APP production. Modulation of a translational regulatory

Homeostatic muffling

SIR — In the brain many mechanisms help to minimize local ionic changes in response to a sudden change in neuronal activity. We suspect that our understanding of these mechanisms has been hindered by the inappropriate use of the term 'buffer' to describe them. We propose an alternative to clarify future discussion.

The term buffer was originally introduced to describe the reversible reaction between H^+ ions and weak acids or bases. Its meaning was later extended to cover the chemical buffering of Ca^{2+} , and then, more boldly, to describe the carriage of K^+ by glial cells away from a site of excess production as "spatial buffering"¹. Although this later extension may have been paradoxically justifiable, as K^+ is not chemically buffered², it has led to more extensive use of the term buffer to describe processes other than chemical buffering.

In order to reduce confusion, we propose that all processes minimizing ionic activity changes (and for H^+ and Ca^{2+} these include buffering) be included in the term, 'muffling', and that 'buffering' be confined to chemical buffering.

To exemplify this we describe an experiment in which we used a double-barrelled pH microelectrode to follow extracellular potential and pH in an isolated leech ganglion, as described elsewhere³, and tested H^+ muffling by brief applications of ammonium ions. The addition and removal of NH_4^+ caused biphasic changes of extracellular pH (pH_e) as NH_3 is much more permeant through cell membranes than NH_4^+ . In HEPES-buffered solution the test caused a pH_e swing of 0.34.

When a CO_2 /bicarbonate solution was applied, pH_e increased to over 7.4, and two tests of muffling caused pH_e swings of only about 0.1. Thus muffling power had more than trebled. After acetazolamide application, however, the pH_e swing increased to 0.26, indicating that muffling power is

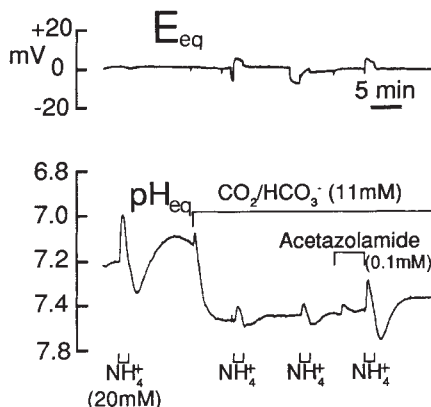
reduced by inhibiting carbonic anhydrase. This confirms earlier reports^{4,5} of a role for carbonic anhydrase in extracellular H^+ homeostasis. So bicarbonate and its rapid

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reduced by inhibiting carbonic anhydrase. This confirms earlier reports^{4,5} of a role for carbonic anhydrase in extracellular H^+ homeostasis. So bicarbonate and its rapid



Potential and pH of extracellular space in an isolated leech ganglion showing responses to ammonium applications.

conversion to CO_2 is important in extracellular H^+ muffling, which will include local buffering, diffusion of H^+ and buffer ions, membrane transport and intracellular muffling, including intracellular buffering.

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Primate perceptions

W. C. McGrew

How Monkeys See the World. By Dorothy L. Cheney and Robert M. Seyfarth. University of Chicago Press: 1990. Pp.377. \$28.75, £19.95.

"MONKEYS seem to be experts at reading each others' behavior, as yet we have little evidence that they are equally expert at reading each others' minds". Thus say Dorothy Cheney and Robert Seyfarth, in as precise and concise a one-sentence summary of a set of findings as anyone could ask. How they got to these conclusions, after years of careful study, makes fascinating reading.

To place *How Monkeys See the World*, one must look at the rise of cognitive ethology in the late 1970s. The theoretical impetus for this school of thought came from the lucid but speculative theorizing of prominent animal behaviourists like Donald Griffin. The empirical testing began with laboratory workers like David Premack and Sue Savage-Rumbaugh extending their studies of captive chimpanzees far beyond questions of whether or not apes have language. What was missing were ways of assessing the intellectual performance of animals in nature, of going beyond observations of behaviour to inferences about the covert mental processes that underlie overt action. This meant grasping tricky issues such as intentionality in the uncontrolled setting of the field.

Seyfarth and Cheney tackled the challenge by turning to field experimentation. They devised ways of empirically testing hypotheses by intervening (as delicately and as minimally as possible) in the ongoing natural lives of their chosen subjects, vervet monkeys living in the Amboseli National Park of Kenya. Such methods have a long history of ethology, and primatologists like Hans Kummer had shown them to be feasible and productive in studies of baboons in Ethiopia. What was needed was a means of systematic interrogation, to elicit knowledge from a monkey going about its daily life, unaware that it was even a subject of testing. This Cheney and Seyfarth did by focusing on the medium of vocal communication, presented through playback of recorded natural calls.

In their simplest form, playback experiments entail recording the responses by free-ranging animals to carefully presented vocal stimuli, for example do resident monkeys approach or avoid the broadcast long calls of their neighbours, depending on whether or not the calls originate from within or without their home range? Seyfarth and Cheney have concentrated more on individual responses,

so that in their best-known, early study they showed the referential content of the vervets' alarm calls given to leopards, birds of prey, and snakes. Each call, given in the absence of its referent, evokes the appropriate anti-predator reaction.

But playback studies can yield much more, sometimes serendipitously: in a study of maternal recognition of offspring's call, they found that not only did a vervet mother attend more strongly to her infant's call, but that at the same time her companions attended to her, indicating their apparent



knowledge of the mother-infant relationship. Again and again, Cheney and Seyfarth devised ingenious ways of 'questioning' their subjects, with the most sophisticated paradigm being one borrowed from developmental psychology. Making use of habituation, one tests the equivalence of two calls by making repeated presentations of one type of call, then by comparing the subject's response on the next presentation, this time to a second type of call. In effect, the amplitude of response to the second type of call with reference to a pre-test control presentation tells whether the monkeys consider the calls to be the same or different.

It is one thing to be knowledgeable about the behaviour of others, both within and across species, and to be able to generalize about their relations. But it is another thing to be aware of one's own knowledge or to accord knowledge to others. It is one thing to use abstract concepts and to communicate them, or to have emotions and beliefs. But it is something else to recognize that others also have such states of mind and are driven by them. In short, monkeys are impressively

intelligent, but they seem to lack a 'theory of mind', to use Premack's phrase.

Had Seyfarth and Cheney stopped with a synthesis of their studies of vervet monkeys, they would have produced a useful research monograph. Instead, they have been more ambitious, in seeking to tie together existing knowledge of such nonhuman primate abilities as deception (widely shown), attribution (present at least in apes), and teaching (apparently absent altogether). Again and again, Cheney and Seyfarth find apes to be superior in intellect, which is not surprising, but they do so in ways that had previously been limited to anecdotal evidence.

In interpreting claims and counterclaims in a contentious area, Seyfarth and Cheney resist easy advocacy arguments and give both sides of the debate. This is especially admirable in a chapter on social and nonsocial intelligence. They are self-declared proponents of the 'social' school of Nicholas Humphrey and Alison Jolly that sees the evolutionary origins of intelligence in the exacting selection pressures of social life. But they give due attention to the claims of the 'nonsocial' school of Katharine Milton, and Sue Parker and Kathleen Gibson that prefers the demands of subsistence as the prime roots of intelligence.

But the work has limitations. Through no fault of their own, Cheney and Seyfarth chose a catastrophically declining population to study, so that their work cannot now be extended or replicated at Amboseli. On another front, their generalizations about apes and monkeys really boil down to chimpanzees and certain terrestrial Old World monkeys. This is more than a pedantic point, as some apes such as gibbons seem to be indistinguishable from monkeys on all measures of intelligence. Finally, as in all lively areas of science, present events have already overtaken even recent conclusions. Though Seyfarth and Cheney repeatedly assert the absence in nonhuman primates of intentional and active teaching (as opposed to passive, inadvertent modelling that allows observational learning), new evidence for both wild and captive chimpanzees suggests that this uniquely human bastion too has fallen, or is at least crumbling.

In conclusion, Cheney and Seyfarth have given us the best-yet exploration of the thinking of another species of primate in nature. As such it stands comparison with an obvious counterpart from the laboratory, Wolfgang Kohler's *The Mentality of Apes* (Kegan Paul, 1925). □

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Diverse functions

M. D. Waterfield

Peptide Growth Factors and Their Receptors. Volumes 1 and 2. By M. B. Sporn and A. B. Roberts. Springer: 1990. Pp.1521. DM996, \$585.80.

THE term growth factor, originally used to describe polypeptides that stimulate cell proliferation, has come to include a variety of molecules affecting growth, differentiation and development. The task of preparing *Peptide Growth Factors and Their Receptors*, the first major treatise on this subject, must have been daunting because of the amazing rate at which new factors and their receptors are characterized.

Progress from discovery of a factor in a tissue extract using a biological assay to definition of the sequence of the active peptide has frequently been a long and complex process which may now often be simplified with the use of recombinant DNA technology. Reviews of progress towards characterization such as found in this treatise serve to emphasize the multifunctional nature of these regulatory peptides. The process of molecular characterization has clarified and simplified the terminology introduced by researchers in their studies. The best example is found in work on the fibroblast growth factor family which now includes related peptides encoded by seven genes previously known by 22 different names and through almost as many biological assays. Thus the number of distinct factors is in a state of flux. During the time since the preparation of this treatise several novel factors have been characterized and receptors for others deduced by cloning and sequencing, a situation that is likely to go on for many years but does not detract from the current value of this excellent summary of our knowledge of growth factors up to 1990. The total number of growth-factor signal molecules remains unknown. Certainly any new treatise will be ten times the size of the present work in five years and will require some new order to be imposed on the field to help organize knowledge about such molecules.

This treatise of 1,500 pages is in two volumes which are divided into four sections. In section A there are two introductory chapters which illustrate the multifunctional nature of growth factors and the methods which have been used to isolate and characterize them. Section B contains detailed reviews of 25 distinct families of factors whose members can be grouped structurally or functionally. The content of each of these chapters falls into a pattern which reflects the gradual accumulation of a series of landmarks in knowledge of the structure-function and biological role of the factors. Epidermal growth factor leads the way, being one of the first discovered and most extensively worked on. Consequently here we can find an ency-

clopaedic summary work on three-dimensional structure, mutagenesis, gene organization, receptor structure and the search for a physiological role in normal and cancer cells. Chapters devoted to platelet, insulin-like, fibroblast and transforming growth factors (TG-Fs), interleukins 1-6 (but not 7-9) and the colony stimulatory factors with erythropoietin complete volume 1.

It is rewarding for those studying these factors to find practical clinical applications described in the chapters on colony-stimulating factors. The discovery and characterization of growth factors has lately been enormously stimulated by the interest of the biotechnology industry and there can be large rewards both in terms of beneficial clinical treatment and financial gain for those who manage to patent clinically useful factors.

The second volume deals first with the cytokines interferon and tumour necrosis factor and then with bombesin and the platelet endothelial factor. Next are reviews of factors which influence nerve cells, including nerve growth factor and a glia-derived nexin. Unfortunately the recent characterization of ciliary neurotrophic factor and brain-derived neurotrophic factor is not included because it has fallen outside the time frame for preparation of this volume. Mullarian inhibiting substance which is another member of the TGF family is covered here as well as the activins and inhibins which have hormonal and growth-regulatory properties. Finally this section covers the somewhat less well known mammary-derived growth factors and the pentapeptide growth factors. Very few factors are not covered most notable perhaps being hepatocyte growth factor and leukaemia-inhibiting factor but these omissions are again due to their recent discovery.

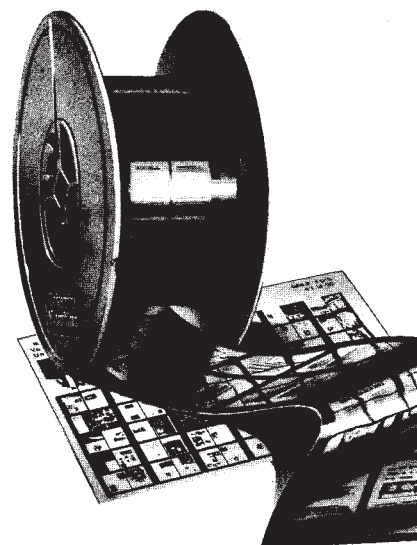
The overall standard of the reviews in section B is first rate and it is wonderful to find a text which can finally be used as a reference book in this area. In some cases the detail is excessive for those not already engrossed in studies on particular factors but for those who want to read about factors in their biological context, sections C and D provide fascinating reading.

Section C includes reviews of the role of growth factors in haemopoiesis, the nervous system, cartilage and bone metabolism, the immune system, and the function of monocytes and macrophages and the extracellular matrix. Continuing in the same theme, section D reviews growth factors and proteases, inflammation and repair, angiogenesis, metastasis, development and oncogenesis. It is amazing that this monumental diversity of subject area is so well covered. This book will therefore fulfil the need for an enormous number of scientists in different disciplines to interpret and collect information on the involvement of growth factors in different areas of biology. □

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Up where the eagles fly

Rory Howlett

Bird Life of Mountain and Upland. By Derek Ratcliffe. Cambridge University Press: 1991. Pp.255. £19.50, \$44.50.

THE upland regions of the British Isles support a splendid and diverse assemblage of birds. In *Bird Life of Mountain and Upland*, Derek Ratcliffe, former chief scientist of the British Nature Conservancy Council and experienced field ornithologist, surveys the distinctive avifaunas of the main upland habitats with a strong emphasis on the ecology and conservation of the species concerned.

Although the book may seem of only parochial interest, this is not so, as many of the issues raised are ones that concern ornithologists, conservationists and ecologists generally. Ratcliffe lists 66 British breeding birds that he considers to have a claim as upland species. (Upland is used here as a collective term for mountains, moorland and hills, of which there are several million hectares in Britain.) These include several spectacular raptors such as the golden eagle, *Aquila chrysaetos*, and the peregrine falcon, *Falco peregrinus*, whose breeding populations are said to be among the highest in Europe. Several species such as the ptarmigan, *Lagopus mutus*, the dotterel, *Charadrius morinellus*, and the snow bunting, *Plectrophenax nivalis*, Ratcliffe regards as 'relict' or 'fringe' species. Thus, "the strong emphasis of northern, tundra-breeding species in Britain and Ireland suggests that our upland avifauna owes its origin mainly to southwards displacement of bird assemblages from the Arctic and Boreal regions during the last glaciation."

Ratcliffe does an excellent job of explaining the relationship between the distribution and relative abundance of upland bird species, for many of which Britain is at the limit of their geographical range, and various environmental influences. Particular attention is given to the effects of climate, geology, human land use (afforestation, for example), availability of suitable habitat and topography. Grouping the upland regions into several main types — the sheep walks, the grouse moors, the deer forests, the fells, the maritime hills and the high tops — Ratcliffe succeeds admirably in giving the reader a flavour of their natural history, bird life and ecology.

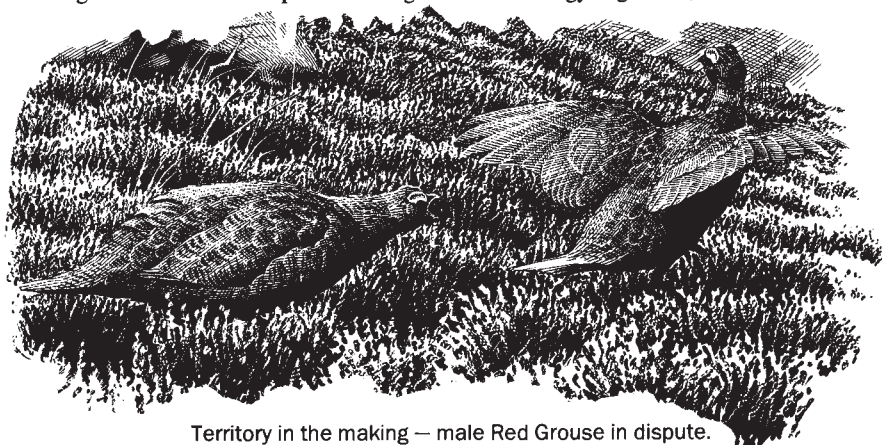
A recurring theme in the book relates to the ecological factors, often mysterious, constraining the distribution of some of the rarer species to limited areas. A case in point is the chough, *Pyrrhocorax pyrrhocorax*, a distinctive member of the crow family more commonly found in Alpine regions. In Britain, the species is confined mainly to sea

cliffs on the western coastlines of Wales and Ireland. According to Ratcliffe, "the chough seems to exemplify a puzzling feature about many birds: they are essentially mobile creatures, potentially able to colonise new ground, yet the distribution of most species is markedly static and there is often an apparent resistance to spread beyond their well-established range." The same point is made in relation to the red kite, *Milvus milvus*, the buzzard, *Buteo buteo*, and the dotterel already mentioned. The ecological reasons for this resistance to spread are largely unknown, underlining the need for further research. Presumably it reflects, in part, the exquisite sensitivity of species' habitat requirements, which, until they are fully understood, will hamper attempts at conservation.

Ratcliffe gives a particularly lucid account of the steady recovery of the peregrine falcon in Britain over recent years, following the dramatic crash in the breeding population resulting from the effects of persistent orga-

demoted to the lowly status of a southern race of the willow grouse, *Lagopus lagopus* — has made it the focus of many scientific studies over the years. After giving a potted history of the grouse shoot, Ratcliffe recounts how in 1911 the Committee of Inquiry on Grouse Disease published 'a lengthy report' blaming epidemics of mortality primarily on caecal infestation by the nematode threadworm *Trichostrongylus tenuis*, exacerbated by the poor quality of heather on some moors. As stated by Ratcliffe, this was one of the first intensive ecological studies of bird species ever carried out; a major recommendation was that there should be rotational burning of the grouse moor to optimize carrying capacity.

As is the case in other species dependent on one or a few food species, populations of red grouse exhibit regular cycles, and studies of their dynamics have played an important role in the development of animal population ecology in general, and our understand-



Territory in the making — male Red Grouse in dispute.

nochlorine insecticides. The crash was first detected by the British Trust for Ornithology in the 1950s. By 1963, when restrictions in the use of dieldrin insecticides began to take effect, the national population was down to 44 per cent of its pre-war level, which had been quite stable. By 1985 the population had made an almost complete recovery, which, according to Ratcliffe, "was final vindication of the case that the persistent organochlorines were the cause of the decline, and the prediction that their reduction as environmental contaminants would be followed by a matching reversal in the trend." Dieldrins have direct lethal effects on adult birds, whereas DDT, which Ratcliffe takes only to have been a secondary factor in the decline, reduces breeding performance through its deleterious effects on egg-shell development. Thus, "Dieldrin residues in Peregrines have declined to very low levels and while those of DDT and DDE are still moderate in some areas, they have not been sufficient to prevent population recovery."

The 'Glorious Twelfth' (of August) is an important date in the British social calendar, marking as it does the beginning of the annual grouse shoot. The economic importance of the red grouse — once celebrated as Britain's only endemic species, but now

ing of population regulation in particular. (Curiously, no mention is made of the work of V. C. Wynne-Edwards, whose thesis that red grouse have evolved adaptations to avoid overpopulation caused great controversy in population ecology because of its dependence on population-level, as against individual-level selection.) Despite decades of research, however, there remain many uncertainties, for example, about the reasons underlying long-term downward trends in the grouse population, and the possible consequences of climate change. Ratcliffe puts it this way: "There is no simple, all embracing explanation of the population changes in Red Grouse. The only general conclusion that can be drawn is that truth has many sides."

Bird Life of Mountain and Upland is a cut above the average bird book, and is the first in what promises to be an outstanding series on bird habitats of the British Isles. Although the book will be of use primarily to those with an interest in the avifauna of Britain, it is also a source of valuable insights into bird ecology and conservation, and would not look out of place on the library shelves of any university zoology department. □

Rory Howlett is Book Review Editor of *Nature*.

Techno farming

Sheldon Krimsky

Plants, Power and Profit. By Lawrence Busch, William B. Lacy, Jeffrey Burkhardt and Laura R. Lacy. Blackwell: 1991. Pp.275. £35, \$39.95.

It is now almost a cliché that agriculture is poised for a new stage in its industrial development by virtue of the scientific advances in biotechnology. The vast machinery of the world's agricultural research and development (R&D) sectors are in a highly competitive race to develop new 'miracle' seeds or other adjuvants that can extract another ounce of productive efficiency from the farm or bring new land under cultivation. The cornucopians are buzzing and the malthusians are watching in disbelief. What about all those projections of food scarcity in the year 2000? Will they have to be recalculated?

Expectations for the role of recombinant DNA technology in agriculture include ecologically safe solutions to pest control, a reduction in the chemicalization of farming towards a more sustainable use of land resources, and the removal of climatic impediments to food production through the redesign of plant genomes. We are not at a point yet where these goals can be evaluated. But we do know that a great deal of promise has been placed on biotechnology. The industrialized nations, and particularly those

strong in the biosciences, have given plant molecular genetics a preferred status in the agricultural R&D sector.

Plant, Power and Profit makes a credible effort to provide a progress report on biotechnology's impact on agriculture. We will need many such efforts by social scientists to keep pace with the momentum of the agricultural research enterprise. This work grew out of a multidisciplinary research project that claims to have garnered over 200 interviews with leaders in all aspects of agricultural research and development. The book tries to do a great deal and can easily be faulted for never quite fulfilling some of its tasks. But the tradeoff, and I believe well worth it, is that its analysis is broad, intellectually provocative, and seeks an integration of science, technology, policy and values.

After an obligatory introductory chapter on trends in biotechnology that gets bogged down in statistics, a second chapter is devoted to sociology and philosophy of science and their relationship to agricultural research systems. A third chapter summarizes a literature that is quite familiar to historians of biology, namely, the origins of molecular genetics and the role of the Rockefeller Foundation in transforming biomedical science. Two additional well-crafted chapters provide an in-depth analysis of the science and political economy of two crops, wheat and tomatoes. The role of biotechnology is discussed briefly because its research applications to these crops are as yet unrealized. Nevertheless, these case studies provide important insights on the origins of technological innovation for these crops and serve as a useful framework for examining other agricultural products. There are no surprises in these analyses. The research effort is responding to new segmented markets (yuppie tastes such as specialty wheat) or to more efficient processing (wheat for easier milling; tomatoes designed for better harvesting). The authors are somewhat sceptical of the myth that in biotechnology we can have an 'efficient' tomato that preserves a semblance of good taste. The essential role of biotechnology, according to the authors, is that "it gives farmers factory-like control over their farming process." For the genetically-engineered tomato it means higher solids and acid content, a uniform colour, and a square shape for machine picking and packaging. All in all, biotechnology is heading toward the complete hegemony of the technological farm.

Two other chapters that address ethical and policy matters are less satisfying because their analysis fails to go far enough. The principal theme is stated succinctly: "We will argue that a 'national biotechnological impact assessment program' should be established. Not just *ex post facto* regulation of social environment or agricultural effects of biotechnology, but also an agenda that would attend to the broader socioeconomic and structural effects that might be expected." Very little attention is devoted to

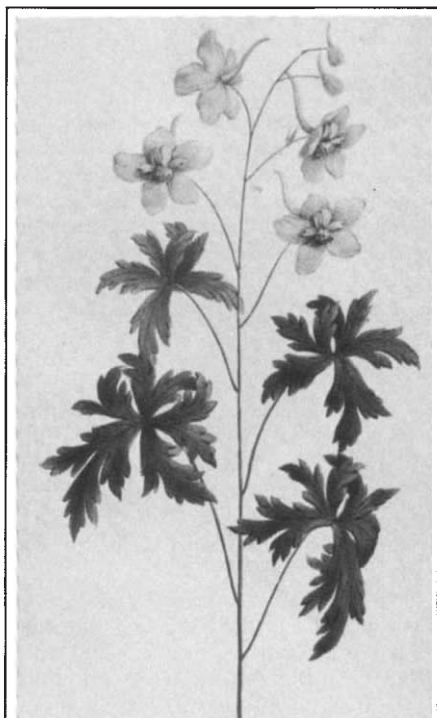
how such a programme would be carried out.

One thing seems very clear. In contrast to other major technological developments, biotechnology is starting out on a new playing field. The social expectations of this technological revolution are different than the past. Until this is understood, the true character and meaning of the public response will be obfuscated. In this new playing field, many of the constraints of traditional crop breeding have been removed. The role of the university and its ties to industry are being re-examined. There are social demands for a new approach to regulation that include more than environmental and health effects. Moreover, the traditional approach of regulating proven hazards has been supplanted by an approach that would regulate speculative or potential hazards. Finally, in this new playing field one dares to select out a process for regulation setting it apart from the long tradition of Euro-American environmental policy that is product-oriented.

Industrial spokespersons are perturbed, quite understandably, by advocates who wish to change the rules of the regulatory process. This has become patently obvious in the case of the controversies over bovine somatotropin (BST) and herbicide-resistant transgenic plants. According to their proponents, if these products do not present a clear hazard to humans or the environment, then what grounds can there be for disapproval? The authors state but do not provide a systematic argument, that such grounds are credible. They call for a "national biotechnological assessment program" in the United States, one that will also help direct the goals of agricultural research.

At stake is nothing less than who controls agricultural technology. Richard Levins and Richard Lewontin argue in their pathbreaking analysis of science policy, *The Dialectical Biologist* (Harvard, 1985), that "The direction of technical change in capitalist agriculture and the research strategies that support this direction are the results of two kinds of factors: the quest for profit by industry and the pursuit of social control by the capitalist class as a whole." Most indicators suggest biotechnology will reinforce this result. Indeed, current state-supported science has an interest in corporate-oriented technological innovation. In the United States this is highlighted by the role the White House Council on Competitiveness has been playing in softening any regulation of biotechnology. *Plants, Power and Profit* makes a valuable contribution to this debate by its careful examination of the sources of innovation in agriculture, by highlighting the contradictory tendencies for the use of agricultural biotechnology, and by daring to pose an alternative, albeit opaque, vision for social governance of agricultural development. □

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Delphinium wellbyi (wild delphinium) found in the mountains of Ethiopia. One of many paintings from Luigi Balugani's *Drawings of African Plants* from the collection made by James Bruce on his travels to discover the source of the Nile 1767-1773 (Balkema, Dfl 285, £89).

Structure and function of telomeres

Elizabeth H. Blackburn

The DNA of telomeres—the terminal DNA–protein complexes of chromosomes—differs notably from other DNA sequences in both structure and function. Recent work has highlighted its remarkable mode of synthesis by the ribonucleoprotein reverse transcriptase, telomerase^{1–4}, as well as its ability to form unusual structures *in vitro*. Moreover, telomere synthesis by telomerase has been shown to be essential for telomere maintenance and long-term viability.

TELOMERES were originally defined functionally on the basis of early cytological and genetic studies which demonstrated that chromosomes with broken ends were unstable (reviewed in refs 1 and 2). The broken ends were able to fuse end to end, leading to dicentric, ring or other unstable chromosome forms. The contrast between this instability and the stability of normal chromosomal ends led to the concept of the telomere as being the specialized structure at the natural end of a eukaryotic chromosome, without which the chromosome is unstable. Molecular analysis has revealed how telomeres carry out another critical function: the ability to allow the end of the linear chromosomal DNA to be replicated completely without the loss of terminal bases at the 5' end of each strand of this DNA. Such loss is predicted from the properties of the machinery of conventional semiconservative replication: its ability to work only in the 5' to 3' direction, and the requirement of the cellular DNA polymerases for an RNA primer (see Fig. 1).

Telomeres have proven so far to be highly conserved among all well-characterized eukaryotic nuclear chromosomes, and to be quite different from the termini of linear viral, non-nuclear plasmid or mitochondrial DNA genomes. Hence, it is useful to define telomeres separately from the much greater variety of terminal structures found at these other DNA ends.

Telomeric sequence organization

Telomeric DNA sequences and structure are similar among otherwise widely divergent eukaryotes. The essential telomeric DNA consists of a stretch of a very simple, tandemly repeated sequence. Examples of telomeric repeat units include AGGGTT for humans as well as other vertebrates, slime moulds and trypanosomes; GGGGTT and GGGGTTTT for the ciliate protozoa *Tetrahymena* and *Euplotes* respectively; AGGGTT (C/T) for the malarial parasite *Plasmodium*, and G_{1–3}T and G_{1–8}A for baker's yeast and the slime mould *Dictyostelium*, respectively (reviewed in ref. 3). A terminal tract of this simple-sequence DNA, typically of a few hundred base pairs in yeast or ciliates, and thousands of base pairs in vertebrates, seems to be sufficient to maintain a stable telomere^{4,5}.

Although the simple telomeric repeats do not conform to a specific consensus sequence, they have a G-rich strand with an orientation specificity with respect to the end of the chromosome. At each chromosomal end, the G-rich telomeric DNA strand runs 5' to 3' towards the terminus and protrudes 12–16 nucleotides beyond the complementary C-rich strand, at least in the various species in which analysis has been possible^{6,7} (Fig. 2).

A loosely defined group of repetitive sequences called telomere-associated sequences are commonly located adjacent and internally to the telomeric sequences. Various telomere-associated sequences are found in species ranging from yeast to humans, and include a wide variety of species-specific sequences, lengths and complexities. Although often found on several chromosomes in a species, such telomere-associated sequences are often absent altogether (reviewed in refs 1, 2 and 5). They do not seem to be essential for chromosome stability and their functional and evolutionary significance is unclear.

Telomerase

The G-rich strand of telomeres is synthesized by the ribonucleoprotein enzyme telomerase (reviewed in ref. 1). Telomerase activities have been identified *in vitro* in ciliate^{8–13} and human¹⁴ cell-free extracts. A synthetic G-rich telomeric DNA oligonucleotide^{10–15} or a telomere^{7,16} is elongated by polymerization, in the 5' to 3' direction, of deoxynucleoside triphosphate substrates into tandem repeats of the telomeric sequence of the species from which the telomerase was made. The reaction does not require ATP. The telomerase RNA and protein components both seem to be essential for activity. The telomerase RNAs of *Tetrahymena* and *Euplotes* have been identified, and contain a sequence, 5'-CAACCCCAA-3' and 5'-CAAAACCCCAA-3', respectively, which is complementary to the telomeric repeats synthesized by the enzyme^{8,9}. Studies *in vitro* indicated that this complementary RNA sequence acts as the template for synthesis of the G-rich telomeric DNA strand^{8,9}. That the 5'-CAACCCCAA-3' sequence in *Tetrahymena* is the template for telomere synthesis was demonstrated by site-directed mutagenesis. Expression of the mutated telomerase RNA gene in transformed *Tetrahymena* cells results in the synthesis of telomeres whose sequence corresponds to the mutated template sequence¹⁶. Telomerase can thus be defined as an unusual ribonucleoprotein reverse transcriptase whose RNA template is an intrinsic part of the enzyme. The current model^{8,9} for the mechanism of telomere DNA synthesis by telomerase is shown in Figure 3.

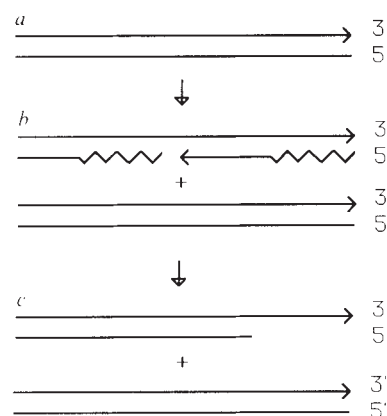


FIG. 1 The problem of complete replication of a linear DNA molecule by conventional DNA replication. *a*, DNA duplex, whose end is on the right, with 5' and 3' ends of each strand indicated. *b*, Parental DNA is copied by a replication fork moving from the left toward the end of the molecule. Leading strand 5'-to-3' synthesis copies the bottom parental strand all the way to its last nucleotide. Discontinuous lagging strand synthesis, also in the 5'-to-3' direction, copying the top parental strand, is primed by RNA primers (zig-zag lines). *c*, RNA primers are removed, and the internal gaps are filled in by extension of the discontinuous DNA and ligation. A 5' gap in this newly synthesized strand is left because there is no primer allowing it to be filled in. Successive replication rounds will produce shortened daughter chromosomes.

In contrast to the sequence specificity of binding by the structural telomere proteins (see below), telomerase accepts as primers a variety of G-rich sequences^{10,11,13-15}. A + T-rich, C-rich, or random sequence oligonucleotides are generally not used as efficiently by telomerase as primers *in vitro*. Strong evidence that telomerase recognizes various non-wild-type telomeric sequences *in vivo* as well as *in vitro* comes from experiments in which telomerase RNA genes with altered template sequences directed synthesis of several tandem GGGGTC¹⁶ or irregular G₅₋₈TT repeats (G.-L. Yu and E.H.B., unpublished work), instead of the wild-type GGGGTT repeats. These results directly demonstrate that telomerase can use a non-wild-type telomeric sequence immediately preceding the added sequence for priming *in vivo*, in agreement with the results *in vitro*.

Compelling evidence that telomerase activity is essential for long-term viability of *Tetrahymena* comes from analysis of telomerase RNA mutations *in vivo*. Overexpression of one particular mutant telomerase RNA gene in *Tetrahymena* is sufficient to cause a dominant negative phenotype characterized by telomere shortening and senescence¹⁶. Cell rescue has not been observed except by loss of this mutant RNA gene through recombination and/or segregating out the plasmid bearing the mutant gene¹⁶ (J. Bradley and E.H.B., unpublished results). In yeast, loss of function of a gene essential for long-term viability, *EST1*, causes steady and continuous telomere shortening over several cell generations, and eventually senescence¹⁷. Cell death is preceded by increased rates of chromosome loss. The *EST1* gene encodes a reverse transcriptase-like protein¹⁸. This, together with the identification of the ciliate telomerases as specialized reverse transcriptases^{8,9,16} and the phenotype of *est1*-deficient mutants, indicates that *EST1* is a protein component of telomerase¹⁸. These findings imply that telomerase is essential for maintenance of telomere length and long-term viability in yeast as well as *Tetrahymena*.

Whether telomerase is used to heal broken chromosomes lacking telomeres may vary from species to species. Healing by the apparently direct addition of simple telomeric repeats to a

broken end has been demonstrated in the yeasts *Saccharomyces pombe* and *Kluyveromyces*, in the protozoa *Plasmodium* and *Paramecium*¹⁹⁻²² and for chromosome 16 in humans²³. Using a mutant *Tetrahymena* telomerase RNA to monitor telomerase action *in vivo*, telomerase has been shown to add the first telomeric repeats *de novo* onto the non-telomeric ends generated during the developmentally controlled chromosome fragmentation in the ciliate *Tetrahymena* (G.L. Yu and E.H.B., unpublished results). This provides a precedent for the telomerase in other eukaryotes being able to heal broken chromosomes by direct telomere addition to the broken end. In yeast, healing of broken chromosomal ends often involves recombination of large chromosomal terminal regions, including regions centromeric to the simple G₁₋₃ repeat sequences, onto the broken end²⁴⁻²⁷. But Murray *et al.*²⁸ found that for a plasmid cut by a restriction enzyme so that a *Tetrahymena* telomeric sequence lay near but not at the resulting end, healing occurred by addition of G₁₋₃ repeats, suggestive of telomerase-mediated healing described above for other species.

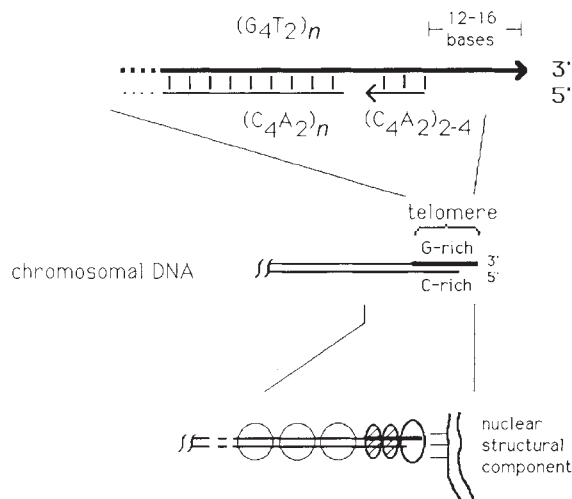


FIG. 2 Eukaryotic chromosomal telomeres. Centre, the orientation of the G-rich and C-rich telomeric DNA strands; top, details of the terminal DNA structure for the *Tetrahymena* telomere. Single-stranded breaks on the C-rich strand occur in the distal part of the duplex telomeric DNA¹, and the G-rich strand (thick line) forms a terminal DNA protrusion; bottom, telomeric proteins and their possible relation to other nuclear protein structural components. Terminally binding telomeric protein (open oval), with an unspecified association with a nuclear structure such as the nuclear envelope of lamins, may be distinct from non-terminal telomere binding protein such as RAP1 of yeast (hatched ovals). The telomeric DNA-protein complex is bordered by nucleosomes^{71,72} (open circles). The drawing represents a composite of information from various species. See text for details and additional references.

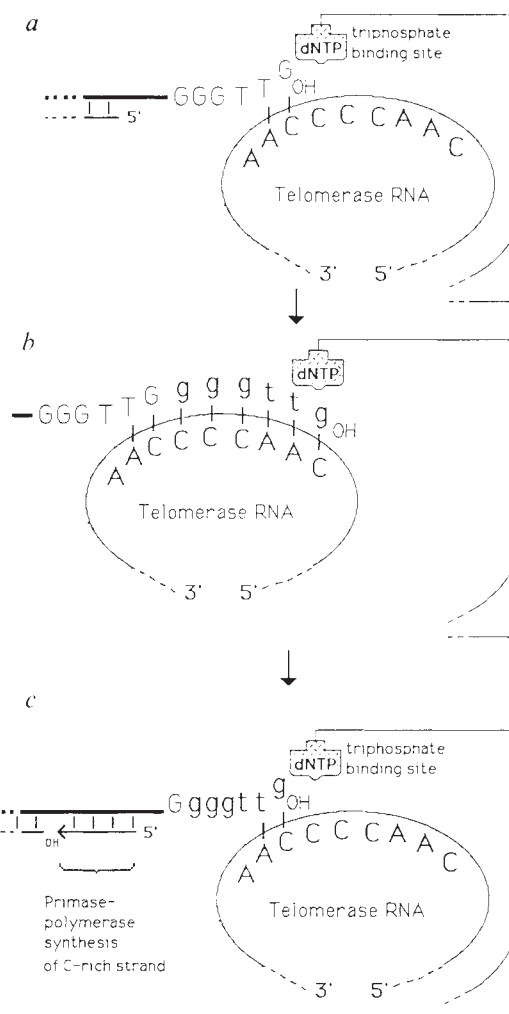


FIG. 3 Synthesis of telomeric DNA by the ribonucleoprotein enzyme telomerase from *Tetrahymena*. a, The 3' few nucleotides of the terminal chromosomal G-rich overhang (see text; thick line, shown arbitrarily as ending in-TTG-3') base-pair with the telomere-complementary sequence in the telomerase RNA.¹ The chromosomal end is extended by polymerization of dGTP and dTTP using the RNA as a template. The relative movement which must occur within the telomerase particle between the RNA template and the polymerization site⁹ during the addition of six telomeric nucleotides is indicated schematically. c, The extended DNA terminus unpairs from its RNA template, becoming available for another round of elongation by telomerase and/or to primase-polymerase^{43,73}. Adapted from refs 8, 9.

Illegitimate recombination has been implicated in processes acting at the simple telomeric repeat sequences in yeast²⁹. But such recombination between terminal telomeric repeats has been detected only in yeast cells transformed by linear plasmids with heterologous telomeres; specifically, its detection depends on assays *in vivo* which select for rescue or increased efficiency of function of the plasmid telomere^{30,31}. Such recombination events have not been detected in yeast cells that have not been selected for these events. Furthermore, telomeric sequences are steadily and inexorably lost in otherwise wild-type *estI*⁻ cells, and most *estI*⁻ cells eventually die¹⁷. Hence recombination-mediated pathways are unlikely to be sufficient for normal telomere maintenance.

Telomeric proteins

The only biological components known to interact specifically with the extreme molecular ends of chromosomal DNA are telomerase and the telomere structural proteins. Although telomeric DNAs have been characterized from a wide variety of species, the protein structural components of telomeres have been identified with certainty in only a few species³²⁻³⁵. The strong conservation of telomeric DNA structure and function predicts that the findings made with these few known telomeric structural proteins will be generally applicable to other eukaryotic species. In the ciliate *Oxytricha*, a heterodimer composed of subunits with relative molecular masses 55,000 and 41,000 (55K and 41K) binds very tightly but not covalently to the overhanging G-rich protrusion, neither protecting nor requiring the adjacent duplex DNA³⁶. The gene for the 55K subunit has been cloned and sequenced; it exhibits some amino-acid sequence similarity with histone H1³⁷. A 50K telomeric protein in *Euplotes* binds similarly³³. Telomere-binding proteins show sequence specificity^{32,33,36,38} and their binding protects telomeric DNA against chemical modification and nucleases^{32,33,36,38,39}. Such proteins are likely to be the 'cap' which protects telomeric ends *in vivo*. It has been suggested³⁹ that these proteins could regulate the action of telomerase *in vivo*, by binding the free DNA end and making it unavailable for further elongation. The main evidence that telomerase is distinct from these structural proteins is that it is not found tightly associated with telomeres, and telomerase activity is released in a soluble fraction from *Euplotes* nuclei lysed in hypotonic buffer (D. Shippen-Lentz and E.H.B., unpublished results), away from the telomere protein fraction³³.

Recent work indicates that the duplex portion of yeast telomeric DNA is bound *in vivo*^{34,35} as well as *in vitro*^{40,41} by the abundant yeast transcriptional regulator protein RAP1. The binding of RAP1 requires a consensus sequence which arises on average every 40 base pairs in the irregular telomeric d(G₁₋₃T) · d(AC₁₋₃) repeats³⁴. There is, however, no evidence that RAP1 binds the presumably single-stranded end of yeast telomeres. Instead, a separate protein analogous in function to the *Oxytricha* terminal protein could fulfil that function (Fig. 2).

Two findings *in vivo* reinforce the idea that the proteins binding to telomeric termini are distinct from those binding internal duplex regions of telomeric sequences. First, foreign telomeric sequences in internal regions of telomeres are stably and indefinitely maintained in yeast and *Tetrahymena*, with no apparent deleterious effects provided that wild-type telomeric repeats are present at the telomeric terminus^{31,42-44} (G.-L. Yu and E.H.B., unpublished work). By contrast, mutated telomeric sequences at the distal ends of *Tetrahymena* macronuclear chromosomes, added continuously by the action of a mutated telomerase RNA gene, greatly impair nuclear division and lead to eventual cell death¹⁶. These results are consistent with a special role for a telomere terminal-binding protein like those of *Oxytricha* and *Euplotes*, which have specificity for the sequence of the G-rich strand overhang³²⁻³⁴, and whose binding would be expected to be impaired by changes in this newly added end sequence. Second, in yeast placing a reporter gene

within a few kilobases of various telomeric ends is sufficient to cause its transcriptional repression⁴⁵. Strikingly, there is no repression if the same gene construct is placed the same distance from a chromosome-internal stretch of telomeric DNA sequence, despite the fact that RAP1 is expected to bind the duplex G₁₋₃T repeat tracts in internal as well as telomeric locations. Again, these results argue strongly that telomeric termini are associated with protein(s) distinct from those binding duplex telomeric repeat sequences. Are these proteins associated with other nuclear components? Telomeres have long been observed cytologically to associate with the nuclear envelope (reviewed in refs 1 and 2). Binding of telomeric termini, directly or indirectly, to such a nuclear structure may be necessary for proper chromosome segregation and hence nuclear division in *Tetrahymena*¹⁶ and possibly other species, and to produce the transcriptional repression effect in yeast.

Telomeric DNA structures

Because of their unusual sequences, telomeric DNAs can assume unusual structures in solution. Double-stranded telomeric DNA is susceptible to rapid digestion with Bal31 nuclease, despite being rich in G and C residues^{5,46}, and when present in supercoiled molecules, can assume unusual structures which are hypersensitive to nuclease digestion, chemical modification and strand invasion^{47,48}. These properties, whose function *in vivo* is unknown, are not unique to duplex telomeric DNAs, and have been found with other duplex G-rich DNA sequences (for example, ref. 49).

The unusual structures G-rich DNA oligonucleotides can assume, and their potential biological properties, have attracted particular attention. Recent work has demonstrated that various telomeric or telomere-like DNA oligonucleotides can form a plethora of G · G base paired structures *in vitro*^{15,39,50-55} (see ref. 56 for a recent review). I will discuss here the two kinds of structures which seem most relevant to telomere function.

First, short G-rich oligonucleotides with the same length and sequence as the 12-16-nucleotide telomeric overhang can assume intramolecular, apparently simple foldback structures, which migrate anomalously fast in non-denaturing gel electrophoresis^{15,50,55}. Some of these oligonucleotides form multiple bands corresponding to discrete intramolecularly folded forms. These structures are probably stabilized by non-Watson-Crick G-G base pairing⁵⁰, but the details are not yet clear (Fig. 4b). Such intramolecular structures merit particular attention, because they are formed by oligonucleotides as short as the presumed substrate for activities or proteins acting on chromosomal termini. They were observed to 'melt' at or below 40-45 °C (ref. 50), well below the melting temperature of the more stable four-stranded forms described next⁵¹.

Second, longer G-rich oligonucleotides containing at least four short runs of G residues can also fold into even more compact forms. These include a very stable, K⁺- or Na⁺-dependent, four-stranded intramolecular structure, a quadruple helix which has as its stabilizing element a planar array of four Hoogsteen-paired G residues⁵⁵ (Fig. 4d). These planar arrays are stacked upon each other, with a central K⁺ or Na⁺ ion between planes. G-rich telomeric DNA oligonucleotides of 12-16 nucleotides are too short to form such intramolecular four-stranded forms. But an intermolecular quadruple guanosine helix built on the same four-stranded structural principal can be formed by association between two intramolecularly-folded short (12 nucleotides) synthetic telomeric G-rich strands⁵⁴ (Fig. 4c).

What is the relationship of the different non-Watson-Crick structures assumed by G-rich DNA to telomere function? Ideas for how telomerase recognizes telomeric DNA primers have focused on structural rather than sequence-specific recognition^{11,50}. *In vitro*, telomerase exhibits specificity for G-rich single-stranded DNA oligonucleotide primers of various sequences. Because the conditions *in vivo* are believed to

resemble those which would thermodynamically favour the stable, K^+ - and Na^+ -dependent quadruple helices described above, the possibility of their having a biological role has been considered. But in considering structures that could be recognized by telomerase, it should be noted that G-rich protrusions of sufficient length to form this type of intramolecular quadruple helix have not been detected in telomeric DNA extracted from eukaryotic cells^{6,7}. There is no evidence that an intra- or intermolecular quadruple helix forms *in vivo*: the patterns of protection *in vivo* from chemical methylation of the terminal nucleotides of *Oxytricha* and *Euplotes* telomeres are reconstituted *in vitro* by binding of their respective telomere binding proteins³⁸, and are quite different from the patterns of protection observed *in vitro* for the four-stranded form⁵⁵. The extreme kinetic and thermodynamic stability of this four-stranded structure formed *in vitro* is difficult to reconcile with a dynamic function *in vivo*³⁹. If such a four-stranded structure formed, it would be expected to persist, given its half-life for unfolding, which is of the order of many hours³⁹. Evidence that the four-stranded G-rich structure may not be used *in vivo* comes from the direct demonstration that the intramolecular four-stranded structure is not bound *in vitro* by the telomere structural protein, and is disfavoured as the substrate for elongation by telomerase (ref. 39; T. Cech, personal communication; M. Lee and E.B., unpublished).

Although the existence of the highly stable four-stranded G-rich DNA forms *in vitro* is well documented, the situation is reminiscent of Z-form DNA, a distinct and defined DNA form whose function *in vivo* remains unknown. But the relationship of other, less stable G-rich structures to telomere function remains an open question. Candidates for other G·G paired structures recognized by telomerase could include the less stable simple foldback structures (see Fig. 4b). These have the appeal of being only moderately stable and hence able to unfold *in vivo* in response to the need to be replicated or to be bound by telomeric structural proteins.

Telomere length and loss

The model for telomere replication and maintenance best supported by available data is that the mean number of tandem simple telomeric repeats at each telomere is determined by a balance of processes that lengthen and shorten telomeres^{43,57}. Many different genetic and physiological factors influence mean telomere length and, by inference, this balance in viable cells^{34,58-62}. Without a mechanism to counterbalance it, rounds of semiconservative DNA replication are predicted to lead to progressive shortening of the chromosome from its ends. Such

shortening over many generations is indeed observed in *est1*-mutant yeast¹⁷, and for certain cases of broken ends lacking conventional telomeric DNA sequences in *Drosophila*^{63,64}.

Germ-line cells in humans have an average of 10 kb of telomeric AGGGTT repeats at each chromosomal end^{5,65}. Analyses of telomere length as a function of age, either in cells from people of different ages, or as a function of cell division number in primary cultures of human fibroblasts, and in certain cancerous cells, show that mean telomere length gradually decreases with increased age or cell division number⁶⁶⁻⁶⁸. Telomerase activity has been clearly documented in extracts of immortalized (HeLa) tissue culture cells¹⁴. But the data are all consistent with the idea⁶⁶⁻⁶⁸ that telomerase activity may be low or absent in normal mammalian somatic cells. It has been suggested that the observed gradual loss of telomeric DNA could lead to chromosome instability¹⁷ and contribute to ageing and senescence⁶⁶⁻⁶⁹. This is consistent with the results cited above for yeast and *Tetrahymena*. One finding inconsistent with a simple form of this idea for ageing in mammalian systems is that mouse telomeres are very long, probably 5–10 times longer than human telomeres, and are not perceptibly shorter in old compared with young mice⁷⁰.

Drugs and telomeres

The G-rich strand of the telomere is the only essential chromosomal DNA sequence known to be synthesized by the copying of a separate RNA sequence. This unique mode of synthesis, and the special structure and behaviour of telomeric DNA, suggest that telomere synthesis could be a target for selective drug action. Because telomerase activity seems to be essential for protozoans or yeast, but not apparently for mammalian somatic cells, I propose that telomerase should be explored as a target for drugs against eukaryotic pathogenic or parasitic microorganisms, such as parasitic protozoans or pathogenic yeasts. A drug that binds telomerase selectively, either through its reverse-transcriptase or DNA substrate-binding properties, should selectively act against prolonged maintenance of the dividing lower eukaryote¹⁶⁻¹⁸, but not impair the mammalian host over the short term, because telomerase activity in its somatic cells may normally be low or absent. Obvious classes of drugs to investigate are those directed specifically against reverse transcriptases as opposed to other DNA or RNA polymerases, and drugs that would bind telomeric DNA itself. These could include drugs that selectively bind the G·G base-paired forms of the G-rich strand protrusions at the chromosome termini, or agents which stabilize an inappropriate G·G base-paired form, preventing it from adopting a structure necessary for proper function *in vivo*. Telomeres have been described as the Achilles heel of chromosomes^{67,69}: perhaps it is there that drug strategies should now be aimed.

Note added in proof. The inactivity of telomerase on four-stranded DNA is described in ref. 74. □

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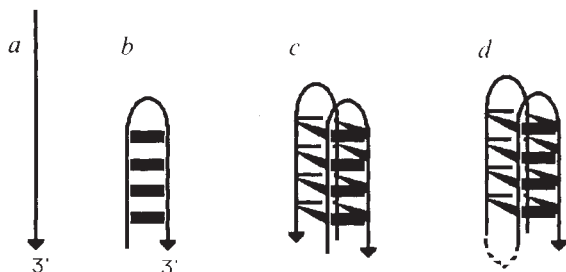


FIG. 4 Some of the structures adopted in solution by G-rich telomeric oligonucleotides containing two (a–c) or four (d) clusters of G residues. a, Single-stranded form. b, Two-stranded foldback form stabilized by non-Watson-Crick G·G base pairs (horizontal bars), present at low and high salt concentrations and visualized as a fast-migrating species in non-denaturing gel electrophoresis⁵⁰. c, Monovalent cation-stabilized intermolecular quadruple helix: a dimer made up of an association of two foldback forms seen in b in antiparallel strand orientation, held together by additional G·G base-pairing between the two intramolecular foldbacks⁵⁴. d, Monovalent cation-stabilized intramolecular quadruple helix: the same structural form as in c but with a continuous polynucleotide chain connecting the two halves of the molecule⁵⁵.

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ARTICLES

Inadequacy of effective CO₂ as a proxy in simulating the greenhouse effect of other radiatively active gases

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The use of an 'effective' CO₂ concentration to simulate the combined greenhouse effect of CO₂ and the trace gases CH₄, N₂O, CFC-11 and CFC-12 is open to question, because the radiative-forcing behaviour of CO₂ is very different from that of these other gases. Model simulations show that different radiative forcing can lead to quite different climatic effects. The thermal infrared opacity of these trace gases therefore needs to be explicitly accounted for when attempting to predict the climate response to increasing concentrations of greenhouse gases.

ATMOSPHERIC CO₂ is currently increasing at 0.5% per year, and the increase is expected to continue. Increasing CO₂ will enhance the greenhouse effect and lead to a global warming¹. The atmosphere also contains the trace gases CH₄, N₂O, CFC₁₁ (CFC-11) and CFC₁₂ (CFC-12), whose concentrations are increasing at 0.9, 0.25, 4 and 4% per year, respectively^{1,2}. These trace gases have strong absorption bands at 8–20 μm^{3,4} in the infrared, and their concentration increases can further augment the CO₂ greenhouse effect^{5–9}. In addition, the trace gases are chemically active; their increases can perturb atmospheric O₃ with subsequent climatic effect^{2,10}. But the spatial distribution of atmospheric opacity which absorbs and emits the long-wave

radiation is different for CO₂ and for the trace gases; for example, CFC₁₃ is optically thin whereas CO₂ is optically thick. This difference can lead to a different distribution of radiative forcing^{6,11}, which in turn will affect the dynamics with subsequent different climate responses.

Here we concentrate on two issues related to the greenhouse effects of CO₂ and trace gases: the extent to which radiatively active trace gases maintain the present climate, and how they differ from CO₂ in affecting the climate. Our model simulations indicate that trace gases provide an important radiative energy source for the present climate system on Earth. The results also indicate that the different infrared opacity of CO₂ and the trace gases can lead to different climatic effects.

Atmospheric general circulation model

Study of the climatic effects of trace gases has so far been based on the use of simple energy balance models^{5,10,12}. Because of the model simplifications such as vertical lapse rate adjustment and fixed relative humidity, the differences in the temperature responses arising from differences in the radiative forcing between CO₂ and trace gases cannot be properly addressed in the energy balance models. General circulation models (GCMs), on the other hand, are based on numerical solutions of the fundamental equations governing dynamical and physical processes in the atmosphere and are more appropriate for studying the effects of radiation on atmospheric dynamics. Most GCMs neglect the trace gases^{13–17}, and the only GCM that explicitly includes them¹⁸ did not study the differences in climate responses between CO₂ and these gases. Excluding trace gases will affect

TABLE 1 Radiative forcing and climate statistics

Case	T_s (K)	ΔT_s	P (mm per day)	Climate statistics			Q (mm)	ΔQ	Radiative forcing (Wm^{-2})				
				ΔP	C	ΔC			STS	UTS	LTS	S	TSS
A*	282.8	—	3.01	—	0.67	—	17.4	—	—	—	—	—	—
B†	287.0	4.2	3.26	0.26	0.65	-0.019	22.6	5.18	-1.06	1.09	1.47	0.950	3.51
C‡	288.0	5.2	3.34	0.33	0.63	-0.033	24.0	6.64	0.742	1.52	0.804	1.63	3.96

Annual and global mean values of radiative forcing simulated in the community climate model and the subsequent changes of climate statistics.

The climate statistics are surface air temperatures T_s , precipitation P , cloud cover C and column water vapour Q . The changes of radiative forcing are for the stratosphere (STS; <150 mb), the upper troposphere (UTS; 150–500 mb), the lower troposphere (LTS; 500-mb surface) and on the surface (S) as well as in the troposphere–surface system (TSS).

* 330 p.p.m.v. CO_2 without trace gases.

† 660 p.p.m.v. CO_2 without trace gases.

‡ 330 p.p.m.v. CO_2 , 1.7 p.p.m.v. CH_4 , 0.3 p.p.m.v. N_2O , 1 p.p.b.v. (1 part per 10^9 volume) CFCl_3 and 1 p.p.b.v. CF_2Cl_2 , assumed constant throughout the atmosphere.

the control climate simulations with implications for the magnitude of climate feedbacks.

To investigate the differences in the radiative forcing between CO_2 and radiatively active trace gases and their subsequent effects in maintaining the current climate, we adopted version 1 of the community climate model¹⁹ developed at the National Centre for Atmospheric Research. This is a spectral transform model with 15 surface spherical harmonics to represent the horizontal structure of prognostic variables; the horizontal resolution is $\sim 4.5^\circ$ latitude and 7.5° longitude. The model has 12 levels in the vertical using energy-conserving vertical finite differences. Realistic land/ocean distributions and topography are used with the seasonal cycle simulated. Both large-scale condensation and moist convective adjustments are included in the cloud prediction section of the model. Many studies have examined model characteristics such as circulation statistics, cloud radiative forcing and sensitivity of climate simulations to horizontal resolution (see ref. 20 for references therein).

To use the model to study the climatic effects of trace gases, we made two modifications. First, the long-wave scheme, which originally included H_2O , CO_2 and O_3 , was expanded to simulate the radiative effects of trace gases⁶. Second, we adopted a model of the ocean²¹ with a 50-metre mixed layer, which includes parameters for sea ice and meridional oceanic heat flux. Typically, the community climate model including mixed-layer ocean needs to run for ~ 20 model years to reach equilibrium, and the

temperature statistics presented below are the averaged values over the last three years of the model simulations.

Model simulations

Table 1 lists the types of climate experiments and the corresponding annual and global mean values of surface air temperature, total cloud cover, column water vapour and total precipitation. Comparison between cases A and B gives the greenhouse effect to be expected if CO_2 concentrations double, and comparison between A and C reflects the greenhouse effect due to the addition of present-day trace gases.

Case A calculates a mean surface air temperature of 282.8 K, which is $\sim 5^\circ\text{C}$ colder than the observed value. The simulated colder surface temperature is not surprising because the cloud albedo in our climate model would need to be lowered to simulate a warmer present climate²¹. The cloud tuning is necessary because the climate sensitivity depends on the control climate itself²². The surface warming due to CO_2 doubling alone is calculated to be 4.2°C , which is comparable in magnitude to some of the AGCM results¹. The addition of trace gases warms the surface by 5.2°C , resulting in a surface temperature much closer to observation. It is therefore clear that the current level of radiatively active trace gases will increase the surface temperature substantially, and that cloud albedo tuning as mentioned above is no longer necessary. In addition, the tuning of cloud characteristics to fit observations may result in strongly

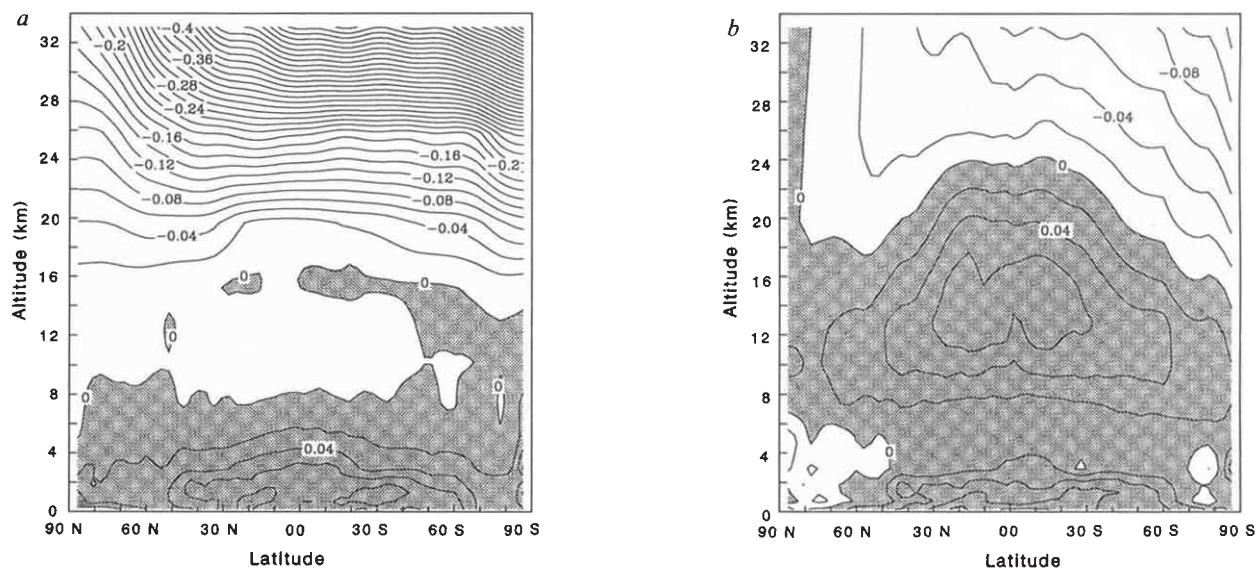


FIG. 1 Latitude-altitude distribution of changes in long-wave radiative heating rate ($^\circ\text{C}$ per day) caused by a, CO_2 doubling and b, the addition of trace gases (see Table 1 for the concentration level of the individual trace gases).

The calculations are based on the climatology on 15 January with an instantaneous increase of the gas concentrations. The shaded areas are warming while others are cooling; the contour intervals are 0.02°C per day.

biased cloud-radiation interactions^{23,24}. The model simulations also indicate that the total precipitation and atmospheric moisture are increased, and the total cloud cover is decreased, key characteristics found in all other GCM simulations¹. For both CO₂ doubling and for trace gases, changes of precipitation and atmospheric moisture are almost linearly proportional to the individual surface warming, but the cloud changes are different.

We first compare the radiative forcing for CO₂ doubling and for trace gases so that it is easier to interpret the difference in the subsequent climate responses. Figure 1 shows the changes in latitude-altitude distribution of the long-wave radiative heating rates for the two cases. Large differences are found in the stratosphere where CO₂ causes large cooling and trace gases induce warming in the lower regions, and in the upper troposphere, especially in the tropics, where a peak warming (>0.07 °C per day) exists for trace gases but not for CO₂ doubling. For trace gases, cooling occurs in the lower troposphere at high latitudes in the northern hemisphere. The differences in the radiative forcing can lead to different temperature responses for CO₂ doubling (Fig. 2a) and for trace gases (Fig. 2b). In the stratosphere, CO₂ uniformly cools the temperature with weak

seasonal and latitudinal dependence, whereas the trace gases generally warm the temperature with stronger seasonal and latitudinal variation. In the troposphere, the temperatures become warmer for both cases, but trace gases provide much larger temperature increases in the tropical upper troposphere in particular, which implies a smaller vertical temperature gradient. This large upper tropospheric warming by the trace gases improves to some extent the cold bias found in case A; for example, the temperature at ~ 10 km in the tropics is warmed by more than 7 °C. But the temperature in this region is sensitive to a variety of other factors, such as convective parameterization²⁵ and cloud radiative properties²⁶, which require further improvements in GCMs.

Differences between the two cases can be clearly seen when we compare the annual and global mean vertical distributions of the radiative heating rate and temperature. Figure 3a compares the heating rates for CO₂ doubling and the trace gases. For illustration, the distribution with twice the concentration of trace gases is also depicted. These comparisons reveal the extent to which radiative forcing of trace gases differs from that of CO₂ doubling and how it will change when the trace gases

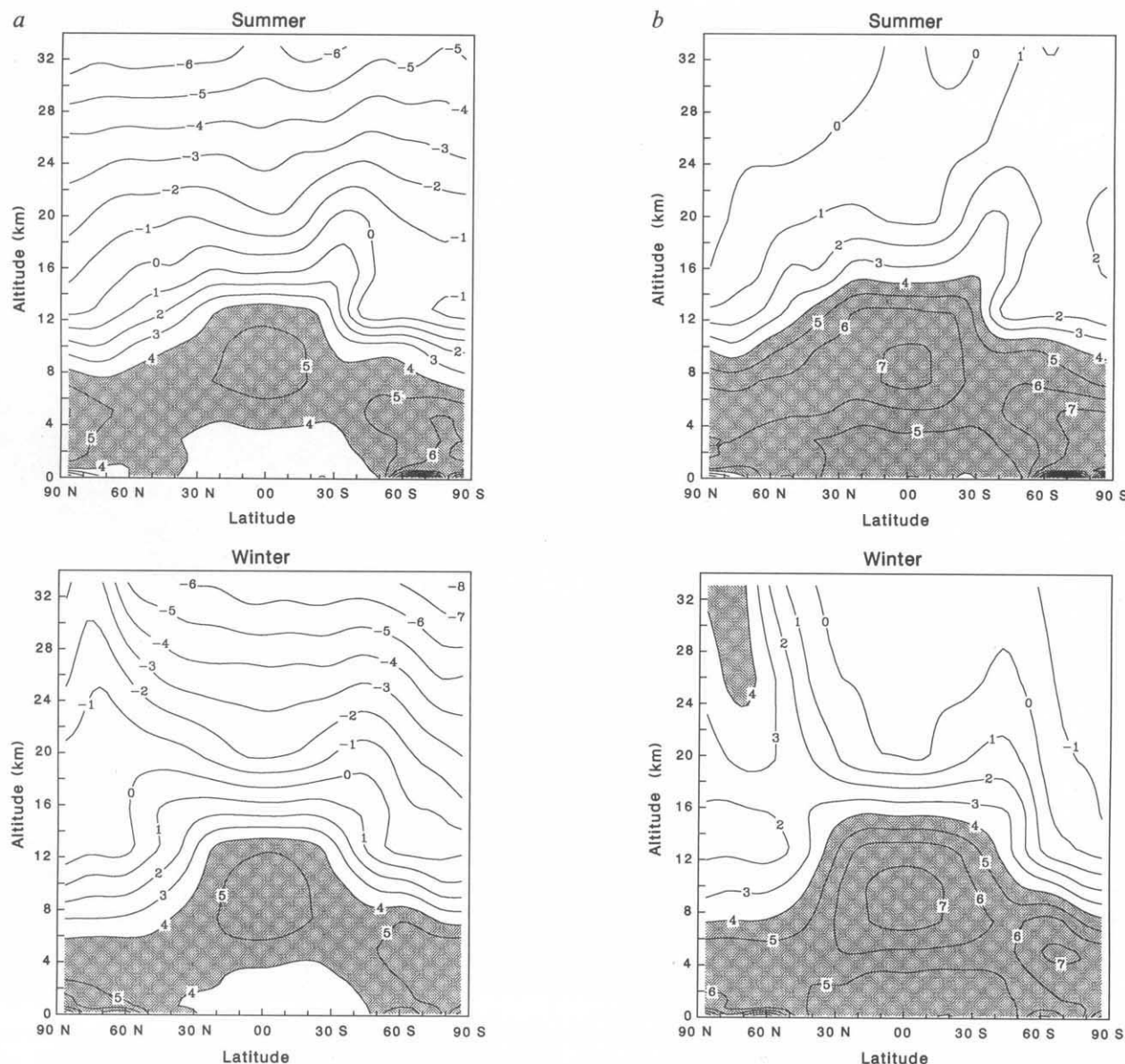


FIG. 2 Latitude-altitude distribution of changes in summer (June–August) and winter (December–February) mean temperature caused by a, CO₂ doubling and b, trace gases. The shaded areas are warmed >4 °C; the contour intervals are 1 °C.

ling and b, trace gases. The shaded areas are warmed >4 °C; the contour intervals are 1 °C.

increase further. The CO₂ radiative heating shows peak warming near the surface, a small amount of cooling at 8–16 km and considerable cooling above 20 km. In contrast, the trace gases provide two warming peaks of similar magnitude (one near the surface, the other at the upper troposphere and lower stratosphere) and little cooling above 22 km. We have evaluated the contribution of the individual trace gases to the total radiative heating: the two warming peaks are mainly caused by equal contributions from CH₄ and N₂O, and to a lesser degree by chlorofluorocarbons. The two warming peaks persist even when the concentrations of the trace gases are doubled. We therefore expect that the change in climate pattern between one and two times trace-gas concentrations will be qualitatively similar to that due to the present level of trace gases.

The changes in annual and global mean temperatures are shown in Fig. 3b. The trace gases increase the temperature from surface all the way up to ~30 km, with peak warming of >6 °C near 8 km. On the other hand, CO₂ doubling produces smaller

warming in the troposphere, and the warming changes to cooling around 18 km. The changes in the vertical temperature profile are fairly similar. But the difference in the tropospheric warming has its smallest value at the surface (~1 °C) and becomes larger as the altitude increases, implying a decrease of the vertical temperature gradient. Similar patterns are also calculated from the seasonal statistics.

Table 1 summarizes the annual and global mean radiative forcing for CO₂ doubling and for the trace gases in the stratosphere, upper and lower troposphere, and the ground separately; the values indicate the distinct difference in the vertical partitioning of radiative forcing. The radiative forcing for the troposphere-surface system is 3.51 Wm⁻² for CO₂ doubling and 3.96 Wm⁻² for trace gases. Results from simple energy-balance models indicated that surface warming was related directly to the radiative forcing in the troposphere-surface system. This led to the notion that perhaps an 'effective CO₂', derived from a linear scaling of the radiative forcing in the troposphere-surface, could be used to simulate the combined greenhouse effect caused by increases of CO₂ and radiatively active trace gases. Our findings indicate that it is inappropriate to do so: the ratio of the surface warming for trace gases (5.2 °C) and CO₂ (4.2 °C) is larger than the ratio of the corresponding radiative forcing in the troposphere-surface system (3.96 Wm⁻² and 3.51 Wm⁻²). This point becomes clearer when we examine the regional differences in surface temperature simulations.

Figure 4 shows the latitude-longitude distribution of the difference in mean surface air temperature in summer and winter for the two cases. The differences are particularly large over land and over the sea-ice boundaries. For example, the trace gases can provide additional warming for the northern United States and Canada of >4 °C during winter and >2 °C during summer. Other land areas that have large differences include South America and central Eurasia. These differences, mainly caused by changes in cloud cover, are statistically significant. Over the sea-ice boundary, the difference is large over Greenland

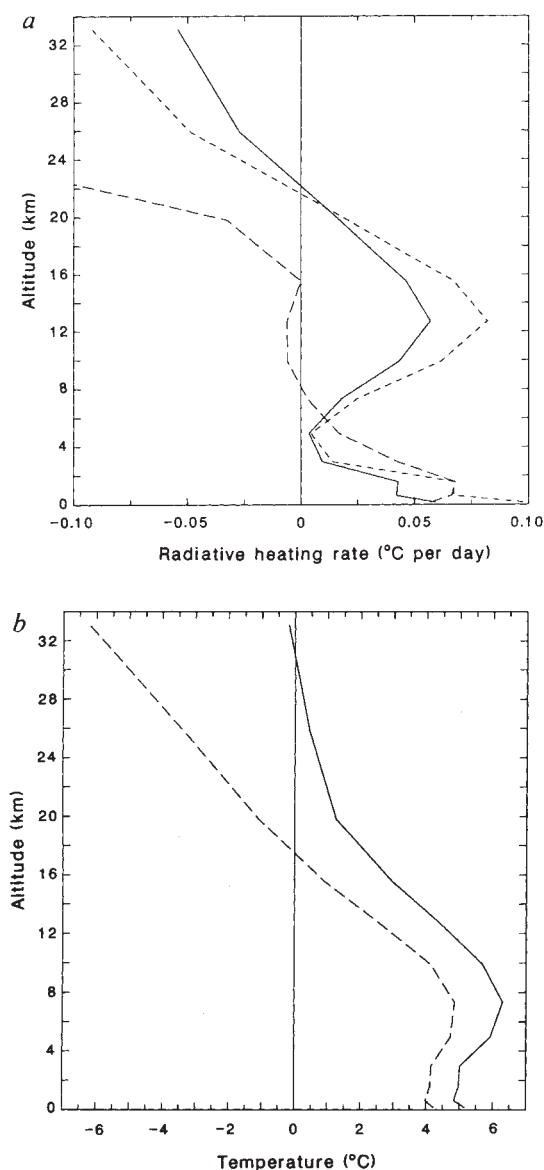


FIG. 3 Vertical distribution of changes in global and annual mean of a, long-wave radiative heating rate (°C per day) and b, temperature (°C) caused by CO₂ doubling (dashed line) and trace gases (solid line). The distributions are calculated by horizontally averaging the values shown in Fig. 1 for heating rates and in Fig. 2 for temperatures. For illustration, the long-wave radiative heating rates calculated using twice the concentration of trace gases (short dashed line) are also shown in a.

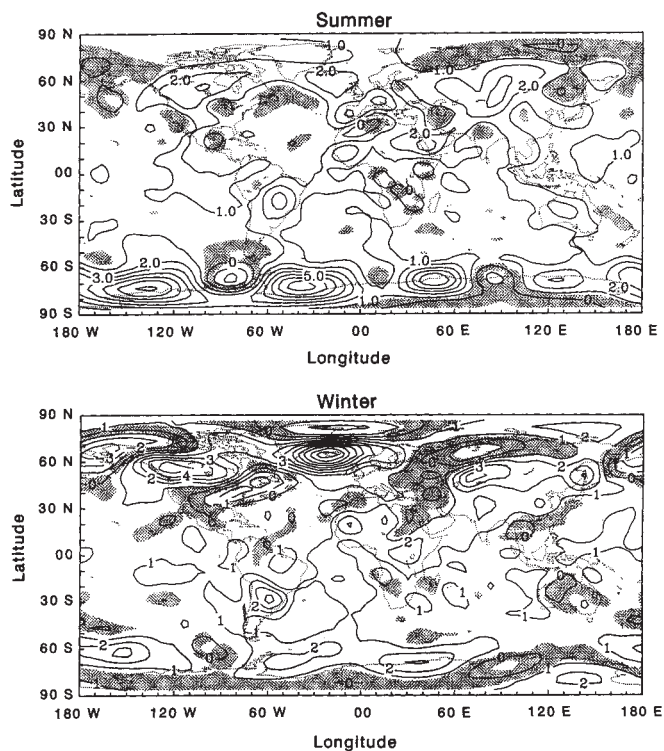


FIG. 4 Latitude-longitude distribution of difference in summer (June–August) and winter (December–February) surface air temperature (°C) between the trace gases and CO₂ doubling. The unshaded areas are the regions where the temperature difference (depicted by contour lines) is significant at 99% confidence level; daily data are used.

during winter because of the ice/snow feedback. Differences over the oceanic areas, however, are fairly homogeneous. These results lead us to believe that increases in trace gases will affect the regional climate differently from CO₂ increases.

Conclusions

The simulations of general atmospheric circulation presented here clearly indicate that trace gases and CO₂ have different effects on the Earth's climate. Two points are specifically noted. First, radiatively active trace gases can provide an important heating source for the atmosphere and should be explicitly included in GCM simulation of the present climate. In our model, the addition of such trace gases yields a realistic simulation of the present climate, correcting the cold bias at the surface and the upper troposphere, especially in the tropics, and the dry bias in the atmosphere. Second, the difference in atmospheric opacity of CO₂ and the trace gases can result in different temperature responses. In the stratosphere, the difference is particularly large: CO₂ increases causes large cooling with a weak dependence on the seasons and latitudes, whereas the trace gases yield a small warming effect with strong seasonal and latitudinal variations. In addition, studies^{2,10} indicate that changes in stratospheric O₃ associated with increasing trace gases can affect the stratospheric temperature. This, together

with the differences in stratospheric temperature responses, implies that the use of GCM simulated stratospheric temperature patterns to identify the signature of the greenhouse effect²⁵ requires a model with explicit treatment of both the trace gases and the climate-chemistry interaction involving O₃. For the tropospheric temperature, the effect of CO₂ and trace gases is very different in the upper troposphere, especially at the tropical tropopause. This is caused mainly by a second warming peak in that region associated with the trace gases. The large warming of the tropical tropopause can decrease the vertical temperature gradient, thus affecting the convection and upper-level moisture with subsequent effect on the life cycle and optical properties of high-level clouds and their feedbacks^{15,16,26}. The simulations also indicate that the difference in the surface warming is significant over the sea-ice boundaries and over land areas because of different cloud responses. On the other hand, the difference over the oceans is small. Consequently, to simulate the regional climate and climate changes due to the greenhouse effect of trace gases requires the explicit consideration of their infrared opacity.

In summary, we conclude from the difference in the temperature responses presented here that it is inappropriate to use an effective CO₂ to simulate the total greenhouse effect of CO₂, CH₄, N₂O and chlorofluorocarbons. □

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I-POU: a POU-domain protein that inhibits neuron-specific gene activation

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A novel, structurally distinct POU-domain protein has been identified that inhibits activation by another positive POU-domain regulator of neuron-specific transcription units. Two *Drosophila* POU-domain proteins, I-POU and Cf1-a, are coexpressed in overlapping subsets of neurons during development. Because I-POU lacks two basic residues in the N terminus of its homeodomain, it cannot bind DNA, but it does form a stable heterodimeric complex with Cf1-a, preventing Cf1-a from binding to DNA recognition elements and from transactivating the dopa-decarboxylase gene. The inhibition by I-POU provides a potential strategy by which the

activation of genes in development is controlled by a homeodomain-containing protein that does not bind DNA.

DEVELOPMENT of all metazoans involves the sequential activation (or inactivation) of a hierarchy of transcriptional regulatory genes. The mechanisms underlying these patterns of activation (or inactivation) are being vigorously investigated (reviewed in refs 1–3). Several mammalian transcription factors and a nematode developmental regulator have been identified that contain a novel structural motif, referred to as the POU domain^{4–11}. The 150-amino-acid POU domain contains a highly conserved N-terminal region of 76 amino acids (the POU-specific domain), a variable linker region, and a 60-amino-acid divergent homeodomain (the POU homeodomain), characterized by the presence of a tryptophan-phenylalanine-cysteine

(WFC) motif in the third 'DNA-recognition' helix¹². The POU-specific domain seems to confer high-affinity site-specific DNA binding and mediate cooperative protein-protein interactions on DNA, whereas the POU homeodomain is critical for DNA binding, and for specific protein-protein interactions¹³⁻¹⁵. Members of the POU-domain gene family are clearly developmental regulators that generate specific cell phenotypes^{10,16,17},

analogous to the functions of homeodomain genes in *Drosophila* early development^{18,19}. For example, *unc-86* is critical for the development of specific sensory neurons in *Caenorhabditis elegans*^{10,17}, whereas *pit-1* is necessary for the generation of three different types of cell in the mammalian anterior pituitary gland¹⁶. The subsequent discovery of many different new POU-domain genes in *C. elegans*, *Drosophila* and mammals suggests

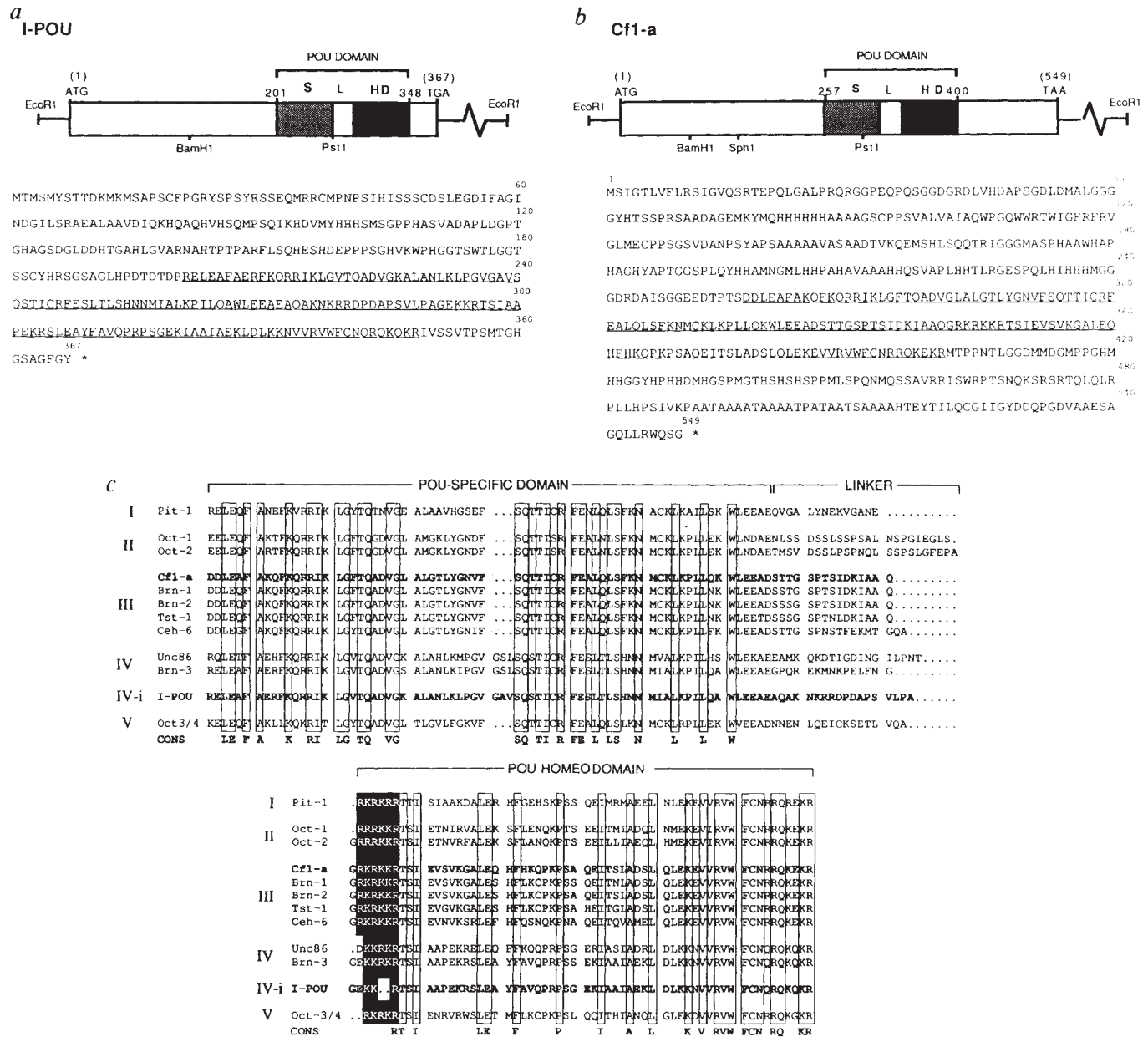


FIG. 1 Predicted amino-acid sequences (single-letter code) of I-POU and Cf1-a and comparison of their POU domains with those of previously characterized POU-domain proteins. *a, b*, Deduced amino-acid sequences of Cf1-a and I-POU are based on multiple independent clonal isolates. Amino-acid sequences are numbered on the right. The POU domains are underlined. For I-POU, the open reading frame (ORF) encodes a 367-amino-acid protein. For Cf1-a, the ORF encodes a 549-amino-acid protein. *c*, POU-specific region the linker region, and the POU homeodomains are labelled, as are the various classes of proteins. The highlighted region indicates the basic cluster N-terminal of the POU homeodomain, depicting the position of two basic residues that are lacking in I-POU. Because of this fundamental structural alteration and the inhibitory function of I-POU, we refer to I-POU as a member of a POU-IV-inhibitory class (POU-IV-i).

METHODS. PCR using fully degenerate oligonucleotides corresponding to highly conserved six-amino-acid regions of the POU-specific domain (S) and

POU homeodomain (HD) were conducted as previously described²⁰. Resultant I-POU and Cf1-a POU-domain probes were used to screen 5×10^5 recombinant plaques from a *Drosophila* head and imaginal disk libraries. Nitrocellulose filters (Schleicher and Schuell) were prehybridized and hybridized in 50% formamide, $5 \times$ SSC, $5 \times$ Denhardt's solution, 50 mM sodium phosphate (pH 7.0) and $100 \mu\text{g ml}^{-1}$ denatured salmon sperm DNA. Probes were labelled by hexanucleotide-primed synthesis to a specific activity of $\sim 1 \times 10^9$ c.p.m. μg^{-1} . Filters were hybridized with 5×10^5 c.p.m. ml^{-1} hybridization buffer for 16–24 h at 42 °C. All filters were then washed to high stringency ($0.1 \times$ SSC, 65 °C). Independent clones were purified and subcloned into pBKSII (Stratagene). Both sense and anti-sense strands of two I-POU and six Cf1-a clones were fully sequenced using oligonucleotide primers and T7 polymerase (Sequenase, USB). G+C-rich regions were also sequenced using Taq polymerase (Taq-track; Promega).

the presence of a large POU-domain gene family that can be structurally divided into five classes (POU-I to POU-V)²⁰⁻²⁷. The coexpression of several different POU-domain proteins in the developing brain in mammals²⁰ raises the possibility that the phenotypic maturation of groups of neurons may be regulated, in part, by combinatorial interactions of POU-domain proteins.

Drosophila provides a model system to study neurogenesis, which results in the production of a highly ordered array of neurons in the central nervous system²⁸⁻³⁰. We therefore studied the expression and function of POU-domain genes in *Drosophila* neurogenesis. Here we report the identification of a functionally novel POU-domain gene product that cannot bind DNA owing to its lack of two basic amino acids in the N-terminal region of its POU homeodomain. This protein, referred to as inhibitory POU (I-POU), is coexpressed in subsets of neurons with another POU-domain protein, Cf1-a, a transactivator of the dopa-decarboxylase (*ddc*) gene. Although unable to bind DNA, I-POU forms a highly stable heterodimeric complex with Cf1-a, and inhibits its ability to bind DNA and activate transcription. Thus it seems that a homeodomain-containing protein may modulate transcription without itself binding to DNA and that the interaction between I-POU and Cf1-a could specify the expression of neuron-specific transcription units in development.

Molecular cloning of I-POU and Cf1-a

Using complementary DNA against total adult *Drosophila* messenger RNA as a template, degenerate oligonucleotides corresponding to two highly conserved amino-acid clusters were designed for polymerase chain reactions (PCR)²⁰ to isolate

putative POU-domain proteins. Two distinct cDNA fragments encoding different POU domains were isolated and used as probes to screen for full-length cDNA clones. From 500,000 recombinant phage of *Drosophila* head and imaginal-disk libraries, several different clones corresponding to each cDNA probe were isolated. DNA sequence analysis of two different inserts (each of about 1.7 kilobases (kb)) corresponding to the first probe (I-POU) revealed an identical open reading frame of 1,101 nucleotides, predicted to encode a protein of 367 amino acids (Fig. 1a). Eleven overlapping cDNA inserts corresponding to the second DNA product of the PCR (initially referred to as *Drosophila* POU-1; DP-1) were analysed. A 2-kb insert from the imaginal-disk cDNA library contained a full open reading frame of 1,647 nucleotides encoding a 549-amino-acid protein (Fig. 1b). After DP-1 had been structurally characterized, a partial *Drosophila* cDNA clone, referred to as Cf1-a, which encodes an identical POU domain, was reported²⁷. We shall refer to DP-1 as Cf1-a. Specific antisera generated against 20-amino-acid synthetic peptides corresponding to the linker regions of each of the two POU-domain proteins, showed that I-POU and Cf1-a proteins migrated to unique immunoreactive bands corresponding to relative molecular masses of 44,000 and 60,000 (M_r 44 and 66K), respectively, identical to the migration of *in vitro*-translated and bacterially expressed I-POU and Cf1-a proteins (Fig. 2c and data not shown).

Structural comparison of I-POU with all the known POU proteins (Fig. 1c) revealed that it is more related to the neuro-specific Brn-3 of mammals²⁰ and Unc-86 of *C. elegans*¹⁰, and thus belonged to the POU-IV class²⁰. It shared 82 and 77% amino-acid identity with Brn-3 and Unc-86, respectively, over

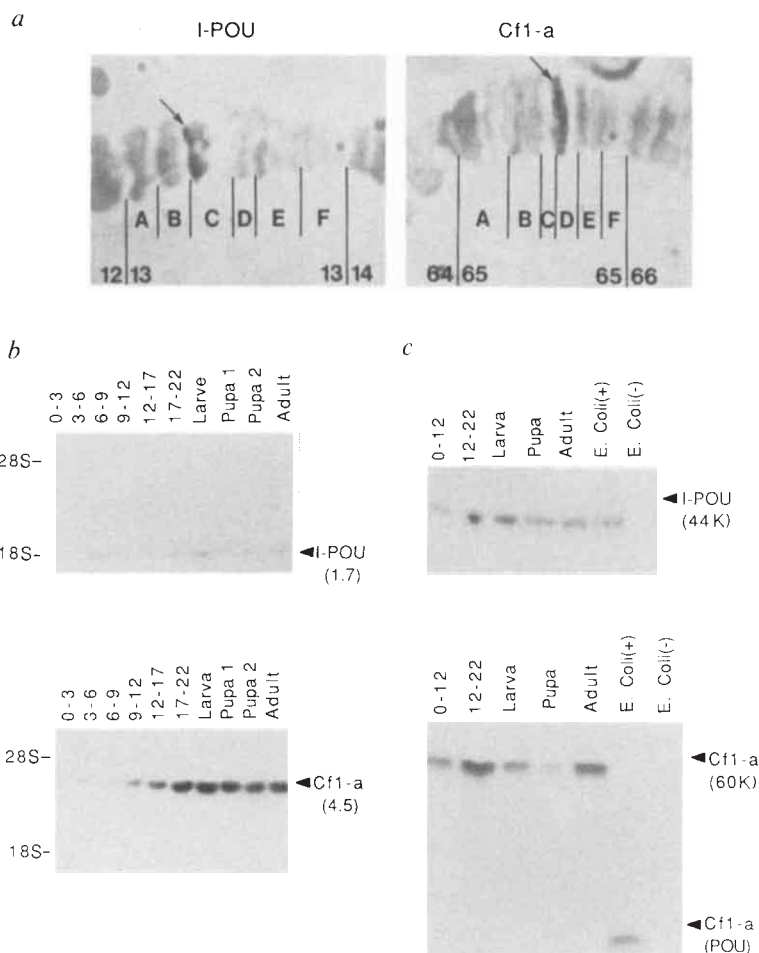


FIG. 2 Chromosomal location, RNA and western immunoblot analyses of I-POU and Cf1-a. *a*, Hybridization *in situ* of I-POU and Cf1-a to larval salivary polytene chromosome squashes. Arrows indicate the hybridization signal. I-POU maps to 13C1-3 on the X chromosome and Cf1-a to 65D1-3 on the left arm of the third chromosome. No other hybridization signals were detected. *b*, Developmental RNA blot analysis. Poly(A)⁺ RNA (5 µg per lane) was isolated from embryos (embryonic stages are given as hours after egg laying), larvae, pupae day 1 (pupa 1) and pupae day 2 (pupa 2) and adult. The nitrocellulose filter was initially probed with labelled I-POU probe (full length, upper) and then reprobed with labelled Cf1-a probe (C terminus, lower). The positions of ribosomal 18S and 28S RNA are indicated, as are the observed 1.7-kb I-POU and 4.5-kb Cf1-a transcripts. *c*, Developmental immunoblot analysis. Extracts were prepared from embryos, larva, pupae and adult. Bacterial extracts prepared from *Escherichia coli* containing an I-POU (pMETI-POU) or Cf1-a (pMETCf1-a) expression vector and the same vector without I-POU or Cf1-a cDNA (pMET; E. Coli(-)) were used as positive and negative controls, respectively. The nitrocellulose papers were incubated with specific antisera against either I-POU or Cf1-a. On the basis of the migration of M_r standards, I-POU antisera specifically detected the presence of a single protein migrating to a position corresponding to an M_r of ~44K, whereas Cf1-a antisera detected the presence of a protein migrating to a position corresponding to ~60K. Selective reactivity with bacterially expressed I-POU and Cf1-a, respectively, confirmed the specificity of the respective antisera. METHODS. Polytene chromosome squashes (Canton-S strain) and RNA blots were performed as previously described^{20,61}. RNA blots were washed to high stringency (0.1 × SSC, 65 °C) and exposed to film at -70 °C for 48-72 h. Protein extracts were prepared and western immunoblot analysis performed as described⁶². Detection involved the use of ¹²⁵I-labelled protein A and autoradiography for 2-5 days. Specific antisera were produced using ~100 µg of I-POU (AQAFNKRDPDAPSVLPAGE)- and Cf1-a (DSTTGSPSIDKIAAQGRKR)-specific peptides, coupled to keyhole limpet haemocyanin. These were used to immunize New Zealand white rabbits. Although effective in immunoprecipitation, neither antibody could

withstand the methanol fixation required for developmental immunohistochemistry.

the entire POU domain (Fig. 1c). But it differed in a characteristic feature of the POU-IV class of proteins in that it lacks two basic amino acids in the N-terminal cluster of basic residues in the POU homeodomain (Fig. 1c). The lack of the codons for these two missing basic amino-acid residues in I-POU transcripts was confirmed by sequencing the products of three independent PCR reactions from distinct batches of *Drosophila* mRNA, and cDNA isolates from two independent cDNA libraries. Cf1-a was best classified with the POU-III class, exhibiting up to 95% homology with three of its mammalian members (Fig. 1c).

Temporal and spatial expression

To determine whether the genomic loci of I-POU and Cf1-a correspond to characterized developmental mutations *in situ* hybridization of salivary gland polytene chromosomal squashes was performed. The two genes were mapped to different loci: I-POU to 13C1-3 on the X chromosome, Cf1-a to 65D1-3 on the third chromosome (Fig. 2a). There are, as yet, no characterized developmental mutations mapping to either of these loci. To assign specific functions to I-POU and Cf1-a, we examined their temporal and spatial expression profiles. RNA blot analysis revealed that I-POU probe hybridized to a 1.7-kb transcript expressed at a relatively constant level throughout *Drosophila* development, with the first detectable transcript appearing at 6 to 9 h of embryogenesis (Fig. 2b). The low abundance of reactive transcripts reflected the highly restricted pattern of I-POU expression (see below). Cf1-a probe hybridized strongly to a 4.5-kb transcript, initially detected at 3 to 6 h of embryogenesis and progressively increasing to maximum levels in late embryo-

genesis, with subsequent slight decrease in expression (Fig. 2b). Immunoblot analysis revealed that, although the amount of I-POU protein remained roughly constant throughout development (Fig. 2c), the amount of Cf1-a protein was maximal at 12–22 h during embryogenesis and in adults, and clearly decreased during larval and pupal stages (Fig. 2c). The apparent discordance between the levels of Cf1-a transcript and protein in larval and pupal stages could reflect post-transcriptional regulation. Thus, I-POU and Cf1-a are expressed in distinct, overlapping patterns.

The patterns of I-POU and Cf1-a gene expression were examined in whole *Drosophila* embryos by hybridization histochemistry (Fig. 3). Analysis *in situ* indicated that I-POU transcripts were first detectable after germ-band shortening (stage 13; as defined by Campos-Ortega and Hartenstein³⁴), and were localized to a subset of neurons in the supraesophageal ganglia and the ventral nerve cord (Fig. 3a). No other tissue stained for I-POU, indicating that the I-POU gene is expressed in a strictly neuron-specific pattern. The subset of neurons expressing I-POU correspond in position and arrangement to the RP1, aCC/pCC and CQ clusters of neurons (median stained cells). The EL cluster and two serotonergic neurons also strongly expressed I-POU (Fig. 3b–e).

The expression pattern of Cf1-a was more complex. Initially, transcripts were detected in the ectoderm of stage 10 embryos (the fourth hour of embryogenesis) at regular repeating intervals, consistent with a segmentation pattern (Fig. 3f). This staining was later colocalized with periodic concavities in the laterodorsal region of the prospective dorsal epidermis in invaginations that

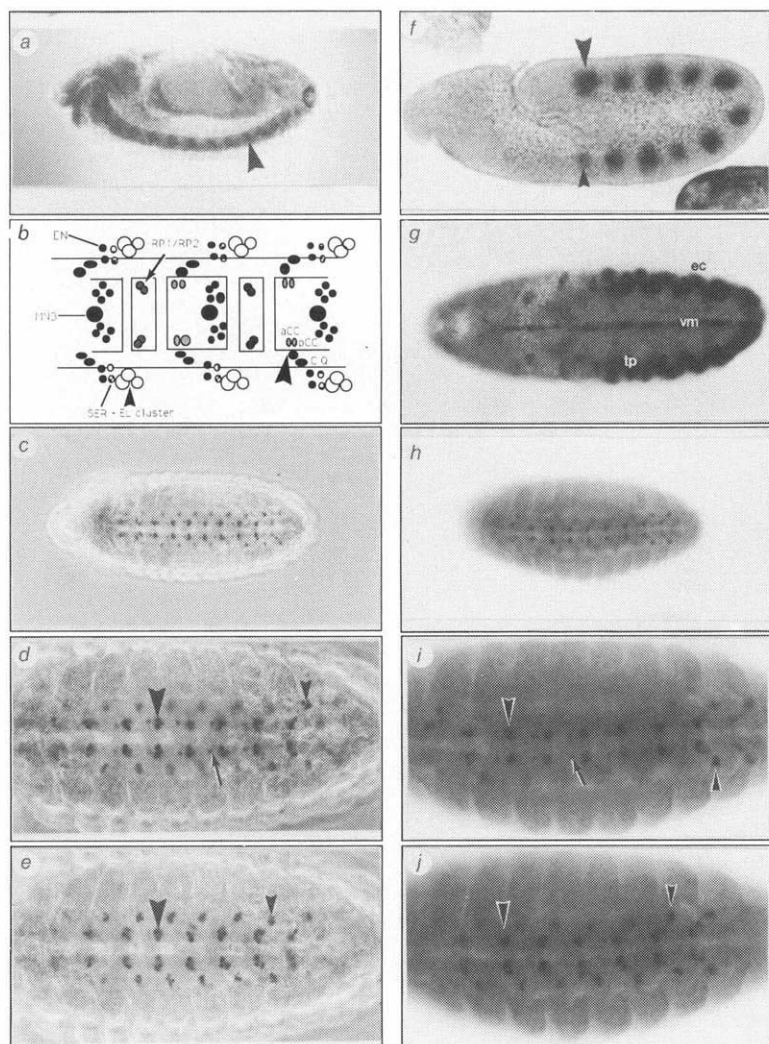


FIG. 3 Spatial patterns of expression of I-POU and Cf1-a transcripts in *Drosophila* embryos. Hybridization *in situ* with digoxigenin-containing I-POU (a–e) and Cf1-a (f–j) probes. Embryo stages are based on Campos-Ortega and Hartenstein³⁴. Anterior is to the left. **a**, Lateral view of a stage 13/14 embryo. I-POU is expressed specifically in a subset of cells in the central nervous system supraesophageal ganglia and ventral nerve cord (vnc) (marked with arrow). **b**, Line drawing of neuronal subtypes, based on Patel *et al.*³⁰, showing medial neuroblasts (MNB), serotonergic neurons (ser) and positions of RP1, RP2, CQ, aCC, pCC and the EL cluster of neurons. **c**, Ventral view of same embryo exhibiting distinct medial and lateral cluster of cells within the vnc that express I-POU. **d, e**, Higher magnification ventral view of same embryo at different focal planes. The positions of the RP1 neuron (thin arrow), the aCC-pCC-CQ cluster (large arrowhead) and the EL cluster and serotonergic neurons (small arrowhead) are indicated. Some cells are slightly out of focus owing to their relative position in the vnc of this whole mount embryo. **f**, Lateral view of a stage 11 embryo. Cf1-a-expressing cells form distinct patches in the dorsal ectoderm which correspond in size and position to the tracheal placodes. Arrows point to the primordia of the anterior (small) and posterior (large) spiracles. **g**, Ventral view of a stage 12/13 embryo. Cf1-a now extends to the ectoderm (ec) surrounding the tracheal pits (tp). The mesectoderm along the ventral midline (vm) also expresses Cf1-a. **h–j**, Ventral view of a stage 14 embryo. Cf1-a is expressed in a subset of neurons in the central nervous system. Most epidermal staining has now disappeared. The positions of the aCC-pCC-CQ cluster (large arrowhead), the EL cluster and serotonergic neurons (small arrowhead) and the RP1 neuron (thin arrow) are indicated.

METHODS. Nonradioactive hybridization *in situ* to whole *Drosophila* embryos was carried out as previously described⁶³ using unique probes from the 5' portion of I-POU and Cf1-a, respectively. Identity of specific neurons was confirmed by immunohistochemistry using specific antibody against Engrailed protein (not shown). Identical patterns of staining were observed in more than 50 embryos examined.

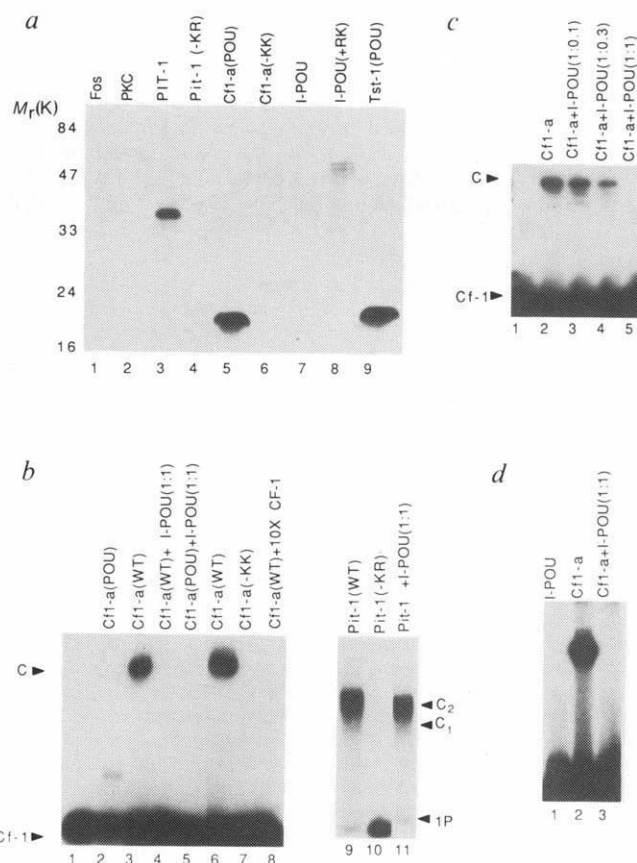
are the origins of the eleven tracheal placodes. At stage 11/12, expression of Cf1-a extended to the epidermis, in positions between the pits (Fig. 3g). This intense epidermal staining, which did not involve gnathal segments, was largely absent in a stage 17 embryo (Fig. 3h-j). Cf1-a was also expressed in a longitudinal strip of cells along the ventral midline of the embryo (Fig. 3g). These cells correspond to the mesectoderm that will generate a mixed population of neurons and glia^{28,29,35,36}, indicating that Cf1-a might be important in specifying a particular neuronal fate. Once the ventral nerve cord and supraesophageal ganglia were formed, Cf1-a was expressed in a subset of neuronal cells (Fig. 3h-j). Examination of these staining patterns revealed that Cf1-a and I-POU were coexpressed in a highly overlapping subset of cells in the central nervous system (see Fig. 3c-e, h-j), with Cf1-a being uniquely, but transiently expressed in the epidermis. Cf1-a was initially identified on the basis of its ability to bind to a neuron-specific *cis*-active element in the enhancer of the *ddc* gene²⁷. Dopadecarboxylase is expressed in the central nervous system and epidermal tissue^{37,38}. Thus, the patterns of Cf1-a expression are consistent with its proposed role in activation of *ddc* gene expression.

FIG. 4 DNA-binding activity of I-POU and Cf1-a. *a*, Random oligonucleotide blot (ROB) analysis of I-POU and Cf1-a proteins. The ability of *in vitro*-translated (or bacterially expressed) proteins to bind DNA was evaluated using a synthetic 35-bp double-stranded oligonucleotide encompassing a contiguous region of 16 bp of complete degeneracy. In the ROB assay proteins used were: c-Fos (lane 1), purified bovine protein kinase C (lane 2, PKC; gift from G. Gill, San Diego) (negative controls), and the *in vitro*-translated proteins wild-type Pit-1 (lane 3), mutated Pit-1 protein that lacked the comparable two basic amino-acid residues in the N-terminal region of the POU homeodomain (Pit-1 [-KR]) (lane 4), wild-type Cf1-a (lane 5), mutated Cf1-a that also lacked the comparable two basic residues in the basic N-terminal region of the POU homeodomain (Cf1-a[-KK]) (lane 6), wild-type I-POU (lane 7), mutated I-POU in which two basic residues were introduced into the homeodomain (I-POU[+RK]) (lane 8) and Tst-1 POU-domain (lane 9). Virtually identical results were obtained using proteins produced in *E. coli*. *b*, Effect of I-POU on DNA binding of Cf1-a and Pit-1 in gel-shift assay. Double-stranded oligonucleotide (30 bp) representing the Cf1 site in the *Drosophila ddc* gene²⁷ was used as the binding site for Cf1-a translated *in vitro*. Lane 1 shows migration of probe in the presence of uncharged reticulocyte lysate. Other lanes show retarded DNA-protein complex (c) with addition of *in vitro*-translated protein representing the POU domain of Cf1-a alone (lane 2) or wild-type Cf1-a (lane 3), whereas addition of an equimolar amount of I-POU to this binding reaction completely abolished the ability of wild-type Cf1-a or Cf1-a POU domain to bind the Cf1 site (lanes 4 and 5). Mutated Cf1-a protein (Cf1-a[-KK]) failed to specifically bind to DNA (lanes 6 and 7); specificity of Cf1-a DNA binding was confirmed by using a 10-times molar excess of the unlabelled Cf1 site (lane 8). Comparable molar amounts of T₃, cyclic AMP or TPA response elements failed to compete DNA binding (not shown). Gel-shift analysis of Pit-1 using the 1P site from the rat prolactin gene¹³ promoter (monomer and dimer binding labelled C₁ and C₂, respectively; lane 9); a mutated Pit-1 protein lacking the two basic residues corresponding to the two amino acids lacking in the I-POU homeodomain (Pit-1[-KR]) failed to bind the 1P site (lane 10). I-POU (1:1 molar ratio) did not interfere with the ability of Pit-1 to bind DNA (lane 11). *c*, Stoichiometry of interaction of I-POU and Cf1-a. A 1:1 molar ratio of I-POU:Cf1-a could completely inhibit Cf1-a binding to its cognate DNA site (compare lanes 2 and 5), and with progressively less inhibition of Cf1-a binding at lower molar ratios (lanes 3 and 4). Migration of probe in the presence of uncharged reticulocyte lysate is shown in lane 1. *d*, Inability of I-POU:Cf1-a heterodimer to bind DNA. ³²P-labelled oligonucleotide encompassing 16 consecutive random nucleotides identical to that used in the ROB analysis, revealed effective formation of DNA-protein complexes with Cf1-a (lane 2), but no detectable complexes were observed with I-POU (lane 1) or Cf1-a in the presence of I-POU (1:1 molar ratios) (lane 3). Comparable results were obtained using bacterially expressed proteins.

METHODS. Proteins were translated *in vitro* using a reticulocyte lysate system, or produced in *E. coli* as previously described¹³. The details of the ROB procedure will be presented elsewhere (H.X. and M.N.T., manuscript in preparation). Briefly, *in vitro*-translated or bacterially produced proteins were electrophoresed on SDS polyacrylamide gels and electro-transferred to nitrocellulose. The nitrocellulose was rinsed in 5% non-fat milk in buffer A

DNA binding activity

As there is a deletion of two basic amino acids in the N-terminal residues of the POU homeodomain of I-POU, it was important to determine whether it could bind with high affinity to any DNA sequences. Deletion mutations in the basic and first helical region of the POU homeodomain of Pit-1 abolish DNA binding, implicating the importance of this region in DNA binding¹³. This is consistent with the evidence of involvement of the comparable N-terminal basic region of classic homeodomains in critical DNA contacts in the minor groove³⁹⁻⁴¹. Initial gel-shift experiments using 10 different DNA binding sites for known POU-domain proteins failed to indicate a binding site for I-POU (data not shown). We therefore used a random oligonucleotide blot procedure to reveal the DNA binding potential of any cloned gene product. This procedure has been successfully used to define specific DNA consensus sites for several POU-domain proteins (H.X. and M.N.T., manuscript in preparation). The procedure involved immobilizing *in vitro*-translated or bacterially produced I-POU on a nitrocellulose membrane, renaturation and incubation with a pool of ³²P-labelled double-stranded oligonucleotides containing 16 consecutive random base pairs. With already known DNA-binding POU-domain proteins, DNA



to renature the proteins¹³ for 10 min. The ³²P-labelled random oligonucleotide, which contained 16 consecutive random positions flanked by *Bam*HI or *Xba*I restriction sites, was annealed, filled and cleaved, analogous to the approach of ref. 64. The nitrocellulose blot and kinased random oligonucleotides (~10⁹ c.p.m. μg⁻¹) were incubated overnight at 4°C in buffer A containing 50 mM KCl. The nitrocellulose filter was washed twice at 4°C in buffer A containing 100 mM KCl, exposed to film for 2-4 h, further washed in buffer containing 300 mM KCl at 4°C, and exposed to film for 4-6 h. Identical data were observed when filters were washed with 100 mM and 300 mM KCl. Gel-shift analysis and mutagenesis was performed as previously described¹³. The Pit-1P site corresponded to that previously used¹³, the Cf1 site was GATGGTCATAATCAAAATTGTAG, flanked by *Bam*HI, *Bgl*II sites.

was retained on the nitrocellulose membrane; with I-POU, no oligonucleotide was retained, indicating that I-POU was unable to bind with high affinity to any DNA site (Fig. 4a). Similarly, using the same oligonucleotides, no binding could be detected by gel-shift analysis (Fig. 4d). Because the two basic amino-acid deletions were the only obvious structural difference between I-POU and other POU-domain proteins, the importance of this basic region in DNA binding was further investigated by deleting two corresponding residues from the POU-domain proteins Pit-1 and Cf1-a, and assessing the resulting mutated proteins for DNA-binding activity. Removal of the two basic residues prevented both proteins from binding to specific DNA sequences (Fig. 4a, b). Conversely, the introduction of two basic amino acids into the POU homeodomain of I-POU allowed the mutated protein to bind DNA (Fig. 4a), indicating that the lack of the two basic amino acids is itself sufficient to account for the inability of I-POU to bind DNA.

Inhibition of DNA binding

Because I-POU and Cf1-a were colocalized in a subset of neurons in the central nervous system, we looked for interactions between the two proteins. Cf1-a bound with high affinity to a double-stranded oligonucleotide corresponding to the Cf1-a site in the *ddc* gene (Fig. 4b), a site required for correct neuronal expression of this gene^{27,37}. Consistent with its inability to bind any DNA sequences, I-POU failed to bind to this recognition element. But when I-POU was mixed with Cf1-a at a 1:1 molar ratio, the binding of Cf1-a was completely inhibited (Fig. 4b; compare lanes 3 and 4, and lanes 2 and 5). This interaction was highly specific because I-POU, at the same ratio, failed to interfere with the binding of Pit-1 to DNA (Fig. 4b, lane 11). The inhibition occurred even at a 0.1:1 ratio of I-POU to Cf1-a,

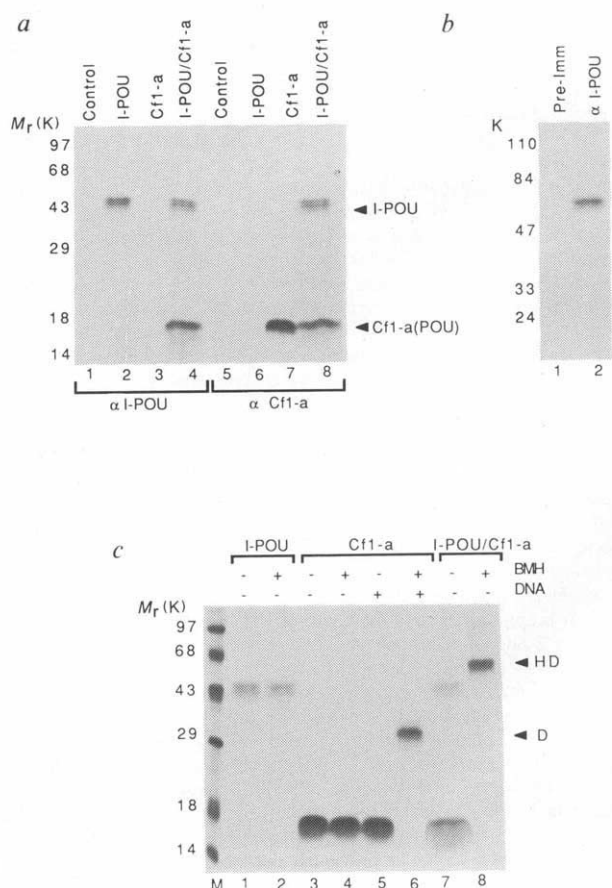
and was complete at a 1:1 ratio (Fig. 4c), suggesting that the stoichiometry of interaction between I-POU and Cf1-a was 1:1 (Fig. 4c). To discover whether I-POU inhibited the ability of Cf1-a to bind any DNA sequences or altered the specificity of Cf1-a binding, as do certain DNA-binding proteins in yeast⁴², we performed gel-shift analysis using the same oligonucleotide containing 16 base pairs of consecutive random nucleotides as probe. Whereas Cf1-a alone could clearly form protein-DNA complexes, I-POU and Cf1-a in a 1:1 ratio could not (Fig. 4d). Thus, the DNA-binding ability of Cf1-a was lost in the presence of I-POU.

Immunoprecipitation of mixtures of *in vitro*-translated I-POU and the POU domain of Cf1-a revealed a strong association between these two proteins in solution. Specific antisera that selectively immunoprecipitated either I-POU or Cf1-a, could coprecipitate both I-POU and Cf1-a from a mixture of the proteins, suggesting protein-complex formation (Fig. 5a). To examine whether this interaction occurred *in vivo*, extracts of adult *Drosophila* were immunoprecipitated with I-POU-specific antisera; the resulting precipitate contained Cf1-a, detected by an antisera specific for this protein (Fig. 5b, lane 2). Cf1-a protein was not observed in extracts precipitated with pre-immune serum (Fig. 5b, lane 1). The dimerization domains of I-POU and Cf1-a were located in the POU domains of both proteins, because the POU domain of I-POU alone can interact with the POU-domain of Cf1-a as efficiently as our wild-type proteins (data not shown).

We confirmed that I-POU and Cf1-a directly interact by chemically crosslinking the *in vitro*-translated proteins using bismaleimidoethane (BMH; Fig. 5c). Addition of BMH to either I-POU or Cf1-a failed to reveal any homodimeric species, suggesting that I-POU and Cf1-a were predominantly monomeric

FIG. 5 Immunoprecipitation and crosslinking of I-POU and Cf1-a. *a*, Immunoprecipitation of I-POU and Cf1-a. Immunoprecipitates with I-POU-specific antisera (α I-POU) are shown in lanes 1–4 and immunoprecipitates with Cf1-a-specific antisera (α Cf1-a) are shown in lanes 5–8. I-POU-specific antisera did not crossreact with any ³⁵S-labelled proteins from lysate programmed with capped control RNA (Pit-1, lane 1). α I-POU specifically precipitated ³⁵S-labelled I-POU (lane 2), but not ³⁵S-labelled Cf1-a (lane 3). When ³⁵S-labelled I-POU and Cf1-a were mixed before immunoprecipitation, both proteins were coprecipitated by α I-POU. α Cf1-a serum did not precipitate any nonspecific protein from programmed lysate (lane 5), or I-POU (lane 6), but effectively precipitated ³⁵S-labelled Cf1-a (lane 7). When I-POU and Cf1-a were mixed before addition of antiserum, α Cf1-a sera precipitated both proteins (lane 8). *b*, Immunoprecipitates from *Drosophila* extract with α I-POU contained Cf1-a. Nuclear extracts were immunoprecipitated with either I-POU preimmune sera (lane 1) or I-POU-specific antisera (lane 2), subjected to SDS-PAGE, electro-transferred to nitrocellulose, and incubated with α Cf1-a. I-POU preimmune sera failed to precipitate any proteins immunoreactive to α Cf1-a (lane 1), whereas α I-POU sera precipitated a reactive protein that migrated to a position corresponding to ~60K, consistent with the size of Cf1-a (lane 2). *c*, Homobifunctional crosslinking of I-POU and Cf1-a. Proteins translated *in vitro* were treated (+) or untreated (–) with 1 mM BMH in solution (lanes 1–4, 7, 8) or in the presence of the Cf1 DNA site (lanes 5, 6). A homodimer(D) was observed for Cf1-a only when Cf1 site was present (lane 6). When equal amounts of ³⁵S-labelled I-POU and Cf1-a proteins were mixed and treated (+) (lane 8) a protein species migrated at the expected size of a heterodimer (HD) following treatment with BMH (compare lane 7 and 8). Similar results were obtained with bacterially produced proteins, with the full-length Cf1-a protein or using I-POU POU domain instead of I-POU holoprotein (data not shown).

METHODS. Immunoprecipitations were carried out essentially as previously described⁴⁹. Briefly, *in vitro*-translated ³⁵S-labelled proteins were incubated in 190 μ l buffer (10 mM Tris pH 7.4), 250 mM NaCl, 5 mM EDTA, 0.25% Noridet P.40 and 0.01% SDS, and 5.0 μ l α I-POU or α Cf1-a antisera and incubated at 4 °C for 1 h. Protein A-Sepharose CL-4B (10 μ l, packed volume) was added and incubated at 4 °C for 30 min. Samples were washed five times in the same buffer, resuspended in SDS sample buffer and heated to 95 °C for 5 min and electrophoresed on a 12% discontinuous SDS-polyacrylamide gel. Crosslinking reactions were performed as previously described⁴³. Briefly, 2 μ l *in vitro*-translated ³⁵S-labelled protein were incubated in 20 μ l buffer (20 mM HEPES, 20% glycerol, 1 mM BMH), incubated



at room temperature for 20 min, and quenched by the addition of 560 mM β -mercaptoethanol before SDS-PAGE.

in solution. When I-POU (M_r 44K) and Cf1-a POU domain (M_r 18K) were mixed in a 1:1 molar ratio, and then crosslinked with BMH, virtually all protein migrated to a position expected for the heterodimer (M_r 62K; Fig. 5c). This suggested that the proteins interact with high affinity and with a stoichiometry of 1:1. Cf1-a, which has high affinity for the Cf1 *cis*-active element (dissociation constant $K_d \approx 2.0 \times 10^{-9}$ M by Scatchard analysis), bound as a DNA-dependent dimer (Fig. 5c, lane 6). Dilution experiments performed after initial mixing of I-POU and Cf1-a revealed that I-POU:Cf1-a heterodimers were fully stable for 24 h, and addition of stoichiometric amounts of I-POU, even after binding of Cf1-a to the Cf1 DNA site, results in rapid and complete disruption of Cf1-a:DNA complexes (data not shown), suggesting that the interactions between I-POU and Cf1-a are of much higher affinity than those between Cf1-a and DNA.

To assess the significance of the interactions between I-POU and Cf1-a, reporter plasmids containing the firefly luciferase gene under the control of the *Drosophila* *ddc* gene promoter or Cf1 elements (see Fig. 6) were cotransfected into CV-1 cells with expression plasmids for both I-POU and Cf1-a. Expression of Cf1-a specifically activated the *ddc* gene promoter to stimulate expression of the reporter gene 15–20-fold (Fig. 6). Mutation of the Cf1 site in the *ddc* gene promoter to a site (mddcL) incapable of binding Cf1-a, abolished the Cf1-a-dependent stimulation (Fig. 6). Additionally the Cf1 element transferred Cf1-a-dependent activation to a heterologous promoter (Fig. 6). Thus, Cf1-a is a transcription factor that can activate the *ddc* gene promoter by binding to a high-affinity *cis*-active element. When I-POU was cotransfected into CV-1 cells with Cf1-a at a 1:1 molar ratio, it virtually abolished the Cf1-a-dependent stimulation of the *ddc* gene promoter, as well as heterologous

promoters containing the Cf1 site (Fig. 6). Thus, the high-affinity interactions between I-POU and Cf1-a inhibit Cf1-a from activating transcription.

Discussion

We have identified a novel POU-domain protein, termed I-POU (for 'inhibitory POU'), that is expressed exclusively in the central nervous system of *Drosophila*. I-POU lacks two basic amino acids vital for DNA binding of the POU domain, and consequently cannot itself bind to DNA. In neurons, I-POU can form a heterodimeric complex with Cf1-a, a POU-domain protein expressed in both the epidermis and central nervous system, and inhibit the action of Cf1-a. On the basis of the ability of I-POU to stoichiometrically form a heterodimer with Cf1-a, we propose that the coexpression of these two proteins in a subset of developing neurons generates a transcriptional switch, inhibiting the ability of Cf1-a to bind to and transactivate specific gene promoters, such as that of the *ddc* gene. Because the amounts of I-POU transcripts and protein are relatively constant throughout development, it is likely that fluctuation in the levels of the positive regulator, or post-transcriptional modifications of I-POU and/or Cf1-a, would act to switch on or off specific programmes of gene transcription. Although the developmental patterns of *ddc* gene expression in some neurons⁴⁴ could reflect the apparent reciprocal actions of I-POU and Cf1-a, it is clear that many *cis*-active elements other than the Cf1 site are important in *ddc* gene activation³⁷.

The inability of I-POU to bind DNA indicates that the basic amino-acid residues N-terminal of the POU homeodomain are crucial for DNA binding. High-resolution NMR of the *Antennapedia* homeodomain and X-ray crystallographic studies of the engrailed homeodomain have supported a model in which

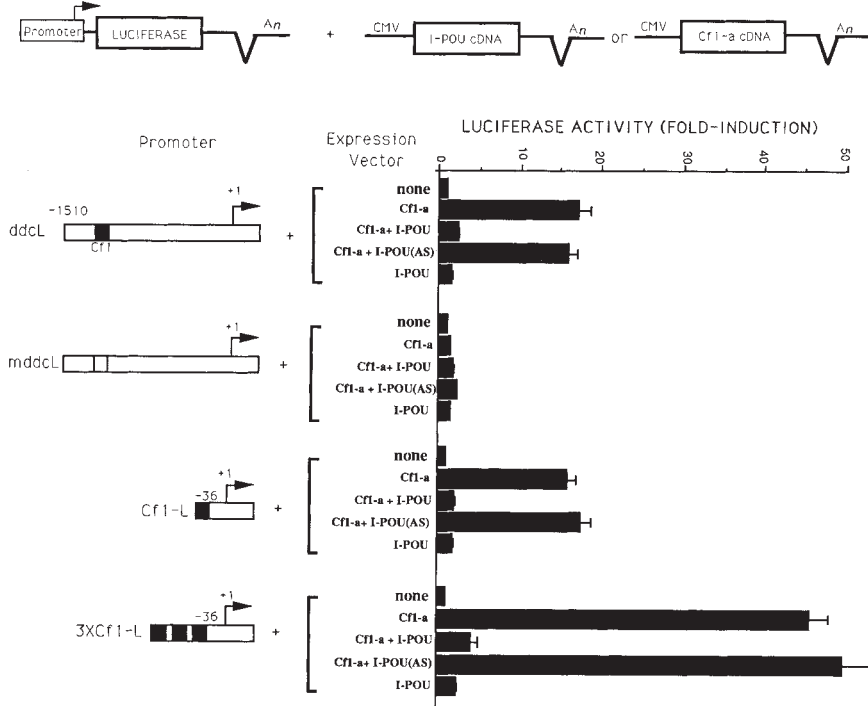


FIG. 6 Cotransfectional analysis of I-POU and Cf1-a. In all cases, CV-1 cells were transfected with 5 μ g reporter gene plasmid and 1 μ g each expression vector. Reporter genes were under control of the dopa-decarboxylase (*ddc*) promoter, or the identical promoter with a mutated Cf1 site (mddcL), or one (Cf1-L) or three (3 $\times$ Cf1-L) Cf1 sites placed 5' of a minimal site prolactin promoter (–34 + 36 bp). These promoters produced detectable luciferase activity when transfected in the absence of expression vectors (between 50–200 light units per 100 μ g whole cell protein). Cotransfection with Cf1-a expression vector (pCMVCf1-a) stimulated reporter gene expression in all reporters, except for mddcL, whereas cotransfection of Cf1-a and I-POU (pCMVI-POU) together at 1:1 molar ratio prevented the Cf1-a-dependent stimulation of reporter gene expression. An expression vector (pCMVI-POU(AS)), in which I-POU cDNA was cloned in the antisense orientation was used as control for effects on Cf1-a-dependent reporter gene expression. Cotransfection of pCMVI-POU alone with ddcL, mddcL, Cf1-L and 3 $\times$ Cf1-L, did not produce any significant reporter gene expression. Results are mean $\pm$ s.e.m. of triplicate determinations. Similar results were obtained in four independent experiments.

METHODS. Transfection of CV-1 cells and luciferase activity measurements were performed as previously described¹³, with analysis performed 24 h after transfection. The mutated site, mddcL, contained a 6-bp cluster mutation of (A $\rightarrow$ C, T $\rightarrow$ G) at the ATAAAT region in the Cf1 site, which completely abolished Cf1-a binding (not shown). Transcription analysis *in vitro* by primer extension for each reporter confirmed correct use of the CAP site (transcription 'start' site). Amount of plasmid was balanced in all experiments by replacing any expression vector not added with pCMVneo plasmid.

three helical structures and two flanking basic clusters are required for DNA binding^{40,41}, and the basic cluster that is distinct in I-POU corresponds to the N-terminal basic cluster of classic homeodomains, which are necessary for making contacts with bases in the minor groove of DNA. Thus, the basic cluster in the N-terminal region of the POU homeodomain probably exerts functions similar to those in the classic homeodomain DNA-binding proteins.

The ability of I-POU to regulate gene expression by forming heterodimers may be analogous to the ability of members of other families of transcriptional regulators to form heterodimers that play a critical part in gene regulation. In the leucine-zipper family of transcriptional regulators, heterodimer formation is required for positive regulation of gene expression⁴⁵⁻⁴⁷. In the ligand-dependent zinc-finger family, heterodimers between members can generate different preferences for DNA binding sites and confer both negative and positive regulation of transcription⁴⁸. The role of heterodimeric complexes in negative developmental gene regulation is well established in regulation of the yeast MAT locus (reviewed in ref. 1). That Id, a mammalian helix-loop-helix (HLH) protein that does not seem to bind DNA, can form heterodimeric complexes with other members of the HLH family (MyoD, E12 and E47) to either inhibit or attenuate their ability to bind *cis*-active elements as homodimeric or heterodimeric complexes⁴⁹, implies the evolution of comparable developmental strategies for both the POU and HLH gene families. Negative regulation in the peripheral and central nervous systems of *Drosophila* might involve

heterodimeric complexes between HLH proteins^{33,50,51}. For example, it has been speculated that *emc* protein can form heterodimers with HLH proteins from the achaete-scute complex^{52,53} and/or *daughterless* locus⁵⁴, to negatively regulate their transcriptional activity, resulting in a suppression of sensory organ development. It is noteworthy that Id and the other putative negative regulators have deletions or alterations of the basic amino acids in the core DNA-binding domains, analogous to the modification that distinguishes I-POU from the other POU-domain proteins. These inhibitory heterodimers, dependent on interactions between nuclear factors, contrast with the negative regulatory strategies based on cytoplasmic partitioning that underlie control of NF- κ B by I- κ B^{55,56}, glucocorticoid receptors by heat-shock proteins^{57,58} and possibly the products of *dorsal*⁵⁹ and the avian oncogene *rel*⁶⁰.

In conclusion, we suggest that I-POU represents a new functional class of POU-domain factors that without binding DNA can modulate gene transcription by negatively regulating specific, positive POU-domain proteins. We refer to this inhibitory class as POU-IV-i (Fig. 1c). Although I-POU fails to bind DNA, its POU domain retains striking homology with those of the positive POU-IV gene regulators, possibly implying additional functions for the POU domain. It is likely that I-POU will be prototypic of other so far unknown POU-domain proteins (and perhaps classic homeodomain proteins) that abruptly terminate the transcriptional effects of specific POU-domain gene activators to control the expression of genes in development. □

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Expression of the homeobox *Hox-4* genes and the specification of position in chick wing development

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The chicken *Hox-4* homeogenes, like those of the mouse, are coordinately expressed in partially overlapping domains during wing development. Local application of retinoic acid, a putative endogenous morphogen, induces *de novo* transcription of *Hox-4* genes. The mirror-image patterns of *Hox-4* gene expression, which are obtained in this way, correlate with the subsequent development of mirror-image patterns of digits. *Hox-4* genes probably encode positional information.

PATTERNING across the anteroposterior axis of the chick limb bud involves a signal from the polarizing region, a small group of mesenchyme cells at the posterior margin of the bud¹. The pattern of the limb is laid down in a proximodistal sequence as cells leave the progress zone, a region of undifferentiated mesenchyme cells at the tip of the bud². Following a polarizing region graft¹ or local application of retinoic acid³, cells at the anterior form posterior structures and this leads to mirror-image duplications that are readily recognized in the digit pattern³. It has been proposed that the signalling of the polarizing region can be understood in terms of the production of a diffusible morphogen that establishes a concentration gradient across the limb bud⁴. According to this model, cells read the local con-

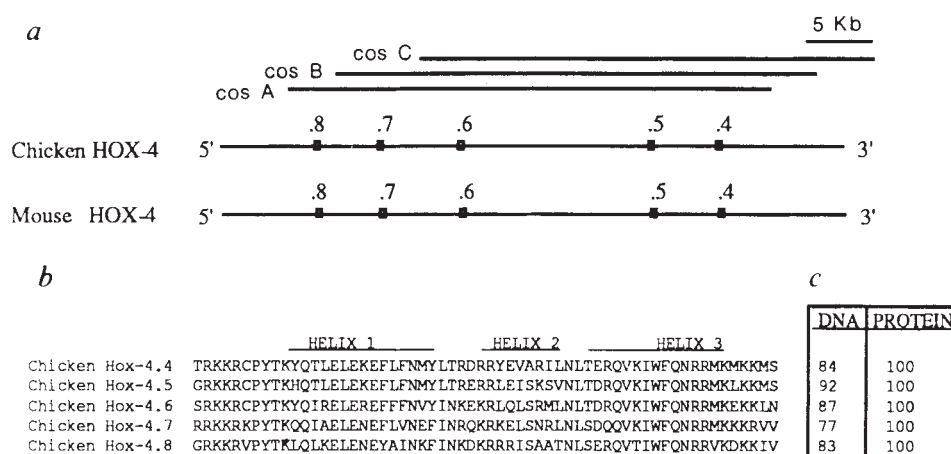
centration of morphogen, possibly retinoic acids⁵, to be informed of their position along the anteroposterior axis.

Vertebrate homeobox-containing genes (*Hox*) from the Antennapedia-like class (class I homeogenes; see, for example, refs 6–9) are good candidates for genes likely to be involved in encoding the position of cells in the embryo. Gene members of the fourth mammalian HOX complex, HOX-4 (previously named HOX-5 (refs 10, 11) are strongly expressed during mouse limb development in striking temporal and spatial patterns^{12–14}. *Hox-4* homeogenes are sequentially activated over a period of 12 h during early limb development. The sequence of activation is colinear with the position of genes in the complex with downstream (3') genes transcribed first and the more upstream (5') genes later. Genes downstream in the complex are expressed more proximally, at the base of the limb, whereas genes located more upstream (such as *Hox-4.7*) are expressed more distally at the tip of the limb bud. The expression domains of all the genes encompass the posterior part of the limb bud tip with upstream genes progressively restricted to this region so that the expression domain of a given gene is included in the domain of the gene immediately downstream of it. The expression of *Hox-4* genes in this anteroposterior pattern is maintained as the bud grows out and therefore cells at varying positions express different combinations of *Hox-4* transcripts¹⁴.

We have now cloned and characterized the chicken counterparts of the mouse HOX-4 complex and show that a similar complex exists in birds and that these genes are expressed in

FIG. 1 Cloning of the chicken HOX-4 complex and homeodomain sequences of five chicken *Hox-4* genes. *a*, Scheme of the organization of the chicken partial HOX-4 complex contained on the three cosmid clones (A, B, C) shown on the top. The filled boxes represent genes, all of them having the same transcription orientation (5' to 3' from left to right). These genes were identified as the chicken *Hox-4.4* to *Hox-4.8* genes by comparison with the mouse cognate complex (shown below). The overall organizations (such as intergenic distances) of both complexes are nearly identical. The *Hox-4.8* gene is probably the last gene of the complex (our results). *b*, Protein homeodomain sequences (single-letter amino-acid code) of the five chicken homeogenes mentioned in *a*. The positions of the α helices (1, 2, 3) are shown on the top. The comparisons of these sequences with their murine cognates are summarized in *c*. The numbers in *c* indicate percentages of identity between mouse and chicken DNA and protein sequences. The chicken homeodomain sequences are identical to their mouse counterparts. These genes were previously named: *Hox-4.4*, -5.2; -4.5, -5.3; -4.6, -5.5 and -4.7, -5.6.

METHODS. A chicken embryonic (stage 21) cDNA library was screened with a mouse *Hox-4.6* cDNA clone under reduced stringency conditions. A partial



cDNA clone of 250 base pairs was thus obtained which contained part of the chicken *Hox-4.6* gene. This clone was then used to screen a chicken cosmid library (Stratagene). Three cosmids were isolated covering 60 kb of DNA. Probes from various mouse *Hox-4* genes were used to hybridize to the digest of the three cosmid clones in order to subclone (bluescript vectors) and sequence²⁴ the corresponding chicken homeoboxes. The positions and orientations of the five genes were determined by conventional strategies.

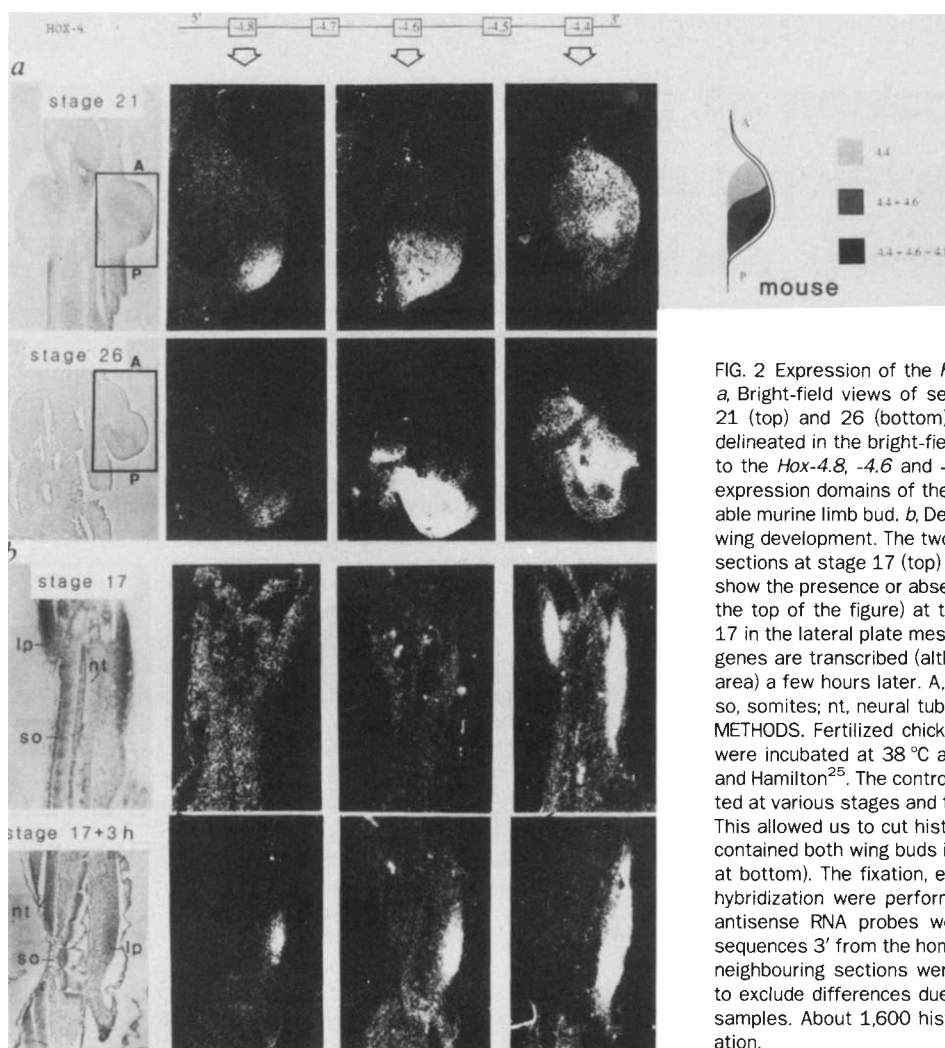


FIG. 2 Expression of the *Hox-4* genes in the developing chicken wing bud. **a**, Bright-field views of sections through chicken embryos at about stage 21 (top) and 26 (bottom). The dark-field panels show enlargements, as delineated in the bright-field views, of the wing bud area after hybridization to the *Hox-4.8*, *-4.6* and *-4.4* probes. The scheme at the right shows the expression domains of the three corresponding mouse genes in a comparable murine limb bud. **b**, Delayed appearance of the *Hox-4* transcripts during wing development. The two left panels show bright-field pictures of embryo sections at stage 17 (top) and 3 h later (bottom). The right dark-field panels show the presence or absence of the *Hox-4.8*, *4.6* and *-4.4* transcripts (see the top of the figure) at these stages. Only *Hox-4.4* is detected at stage 17 in the lateral plate mesoderm, including the limb field, whereas the three genes are transcribed (although *Hox-4.8* is transcribed in a very restricted area) a few hours later. A, anterior; P, posterior; lp, lateral plate mesoderm; so, somites; nt, neural tube.

METHODS. Fertilized chicken eggs (Ross White; from a local UK breeder) were incubated at 38 °C and the embryos staged according to Hamburger and Hamilton²⁵. The control or manipulated (see later) embryos were collected at various stages and then pinned out on a solid support during fixation. This allowed us to cut histological sections of early embryos that routinely contained both wing buds in the same orientation (anterior at top, posterior at bottom). The fixation, embedding in paraffin wax, sectioning and *in situ* hybridization were performed as previously described²². The ³⁵S-labelled antisense RNA probes were synthesized from DNA templates covering sequences 3' from the homeoboxes to prevent cross-hybridizations. Serial neighbouring sections were hybridized with three or five different probes to exclude differences due to the progression of the sectioning within the samples. About 1,600 histological sections were examined after hybridization.

the same pattern as in mammalian limb buds. We manipulated chick wing buds by either grafting polarizing region cells or applying retinoic acid so that anterior cells form posterior structures, and found *de novo* expression of *Hox-4* genes in developing wing buds. When cells are specified to form posterior structures, this is preceded by a change in *Hox-4* gene expression domains and so allows a direct molecular view of the newly established pattern.

The chicken HOX-4 complex

We screened a chicken limb complementary DNA library using a mouse *Hox-4.6* probe. A chicken *Hox-4.6* cDNA clone was thus obtained and used to isolate three overlapping genomic cosmid clones which cover about 60 kilobases (kb) of DNA. This piece of genomic DNA contains most of the HOX-4 complex, from the 3' end of *Hox-4.3* to the 5' end of *Hox-4.8* (Fig. 1a). The exact mapping of the complex revealed an overall organization nearly identical to that in mammals¹¹. The intergenic spaces are conserved as well as the transcription direction of the various genes. A comparison between the chicken homeobox sequences (shown in Fig. 1b) with their murine counterparts gives from 77 to 92% identity at the nucleotide level, and a perfect identity for all the homeodomains in the proteins (Fig. 1c).

Specific antisense RNA probes were prepared for the five different genes (*Hox-4.4* to *Hox-4.8*; Fig. 2a) and their expression pattern analysed by *in situ* hybridization in normal chick wing buds at various developmental stages. At stage 17 *Hox-4.4* is strongly expressed in the wing bud, whereas a signal for *Hox-4.6* is not detected. At this early stage *Hox-4.8* tran-

scripts are not seen (Fig. 2b), but 3 h later, just before stage 18, transcripts from all these three genes can be readily detected (Fig. 2b). At later stages (Fig. 2a), a typical 'Russian dolls' pattern is observed with downstream genes (for example *Hox-4.4*) expressed more proximally and anteriorly than upstream genes (for example, *Hox-4.8*). The expression boundaries overlap in the posterior part of the progress zone (Fig. 2a). Analysis of the other *Hox-4* genes (*-4.5* and *-4.7*) shows that their expression patterns fit into the same scheme (data not shown). Thus the temporal and spatial features of *Hox-4* gene expression are similar in chick and mouse limb buds.

Mirror-image expression patterns

If *Hox-4* genes are involved in the establishment of the chick wing pattern, then any manipulations that lead to changes in pattern should be preceded by a respecification of *Hox-4* gene expression domains. This was confirmed by grafting a polarizing region to the anterior margin of a stage 18 bud. The operation leads to mirror-image duplications of the digit pattern. Cells in the anterior part of the bud now form posterior structures and, instead of the normal 234 digit pattern, the digit pattern is 432234 (Fig. 3a). An analysis of the *Hox-4* gene expression domains 24 h after the graft, well before any distal structures have developed, reveals that a reorganization of these domains occurs in the manipulated bud. In contrast to results obtained with another *Hox* gene¹⁵, a mirror-image duplication of the expression pattern is now observed (Fig. 3b). The new *Hox-4.6* and *-4.8* transcript domains that are now present in what was the anterior part of the wing mimic the normal posterior pattern. The *Hox-4.8* domain is contained within the *Hox-4.6* domain

and the size of the new anterior domains is comparable to that of the normal posterior domains.

Retinoic acid induces ectopic expression

Beads that were soaked in a solution of dimethylsulphoxide containing 0.1 mg ml^{-1} retinoic acid and that were introduced at the anterior margin of stage 18 buds lead to mirror-image duplicated patterns of digits (Fig. 4a). After 24 h of treatment, an ectopic *Hox-4.6* transcript domain is observed in the anterior part of the developing wing, in a region that does not express *Hox-4.6* in normal buds. Three independent examples are shown in Fig. 4b. The ectopic expression domain is not equally distributed around the hole (left by removal of the bead; black arrows in Fig. 4b) but is restricted to a distal subectodermal area. This *Hox-4.6* domain induced by retinoic acid at 24 h is much smaller than the original posterior domain and still is smaller at 48 h, although by then it has started to expand (Fig. 4b). In this experiment, the bead soaked in 0.1 mg ml^{-1} retinoic acid was removed after 24 h. The persistence of the anterior domain shows that respecification of *Hox-4* expression is irreversible. After 72 h of treatment, when the wing shows clear morphological signs of duplication, the ectopic domain has a wide distribution (Fig. 4b).

The respecification of wing pattern by retinoic acid takes place in two steps¹⁶. In the first phase, up to about 12 h, any changes in wing pattern are reversible and normal digit patterns result when beads are removed during this time period. In the second phase between 14 and 24 h, the 'duplication' phase, irreversible changes in pattern are induced. Additional digits are added sequentially with the first extra digit to appear being digit 2 (giving rise to a 2243 pattern). A slightly longer treatment adds digits 2 and 3 (a 32234 wing). By 24 h, the irreversible reprogramming is completed and full duplications will develop.

To determine at what time and in which order the various *Hox-4* ectopic domains appear after retinoic acid treatment, we examined the expression pattern of the same genes as for Fig. 2 at 4, 10, 16, 20 and 24 h of retinoic acid treatment (Table 1). After 4 and 10 h, none of the posterior *Hox-4* genes are ectopically expressed (Table 1), and by 16 h *Hox-4.6* and *-4.8* are still not switched on in anterior mesenchyme. Only *Hox-4.4* could be induced in the very anterior part of the wing in which it is normally not expressed (Fig. 4c). A 20-h treatment resulted in the appearance of the *Hox-4.6* ectopic domain, whereas *Hox-4.8* was still not detected. In all cases the ectopic expression of *Hox-4* genes could be seen after 24 h of treatment, although in

TABLE 1 Time of appearance of ectopic expression domains

Hours of RA exposure	0.1 mg ml ⁻¹ RA			1.0 mg ml ⁻¹ RA		
	4.4	4.6	4.8	4.4	4.6	4.8
4	ND	—	—	—	—	—
10	ND	—	—	—	—	—
16	ND	—	—	—	—	—
20	+	+	—	—	—	—
24	+	+	+/-	—	—	—
48*	+	+	+	+	+	+
40*	+	+	+	+	+	+
72	+	+	+	+	+	+

Time of appearance of ectopic expression domains of *Hox-4* genes in developing wings in which beads soaked in either 0.1 or 1.0 mg ml⁻¹ retinoic acid (RA) were implanted. ND, not distinguishable from the normal domain; +, detected; —, not detected; +/-, detected in some cases only.

* See legend to Fig. 4 or 5.

one bud (shown in Fig. 5a) *Hox-4.8* was still not switched on in anterior mesenchyme at 24 h. The *Hox-4* genes are thus switched on between 16 and 24 h, during the 'duplication' phase. The genes are switched on in anterior mesenchyme in the same sequence and within a similar time as they are switched on normally in the posterior part of the bud.

Dose-dependence

The morphogenetic response of the limb to application of retinoic acid at the anterior margin is not only dependent on time but also on dose¹⁷. Low doses give partial duplication, whereas higher doses (beads soaked in 1 mg ml^{-1} retinoic acid) still give symmetrical digit patterns but anterior digits are progressively lost, giving patterns such as 4334 and 434 (Fig. 5a). At 24 h, the higher dose of retinoic acid had not yet induced a *de novo* transcription of the *Hox-4.6* or *-4.8* genes. The ectopic transcription of these two genes was, however, detected after 40 h (Fig. 5c). The new transcript domains do not appear to be next to the bead but in a more distal position. Very high concentrations of retinoic acid may inhibit expression of 5' genes. This could account for both the distal displacement of the ectopic domains of 5' gene transcripts and the delay in their appearance because time is required for the level of retinoic acid to drop.

The buds treated with the higher dose of retinoic acid are narrower¹⁸ and, at the tip of the limb bud, the ectopic domains for *Hox-4.6* and *-4.8* are continuous with the normal posterior

FIG. 3 The *Hox-4* expression domains after grafting of a polarizing region. a, Central panel shows the scheme of the experiment with stage 18 donor (top) and host (bottom) embryos. The black arrows indicate the wing patterns for both control (on the right) and manipulated (on the left) wings. The digit pattern (234) on the right is normal, whereas the one on the left (432234) is mirror-image duplicated. b, Expression domains of the *Hox-4.8*, *-4.6* and *-4.4* in control and manipulated wings 24 h after grafting. Note the symmetry of the expression domains compared with the symmetry of the digit pattern. The orientation of the samples is as in Fig. 2. PR, polarizing region; A, anterior; P, posterior; h, humerus; r, radius; u, ulna.

METHODS. Grafting was as previously described¹⁵. In each series of experiments, some embryos were allowed to develop for a further 6 to 7 days. They were then fixed and stained to reveal their skeletal patterns²⁶.

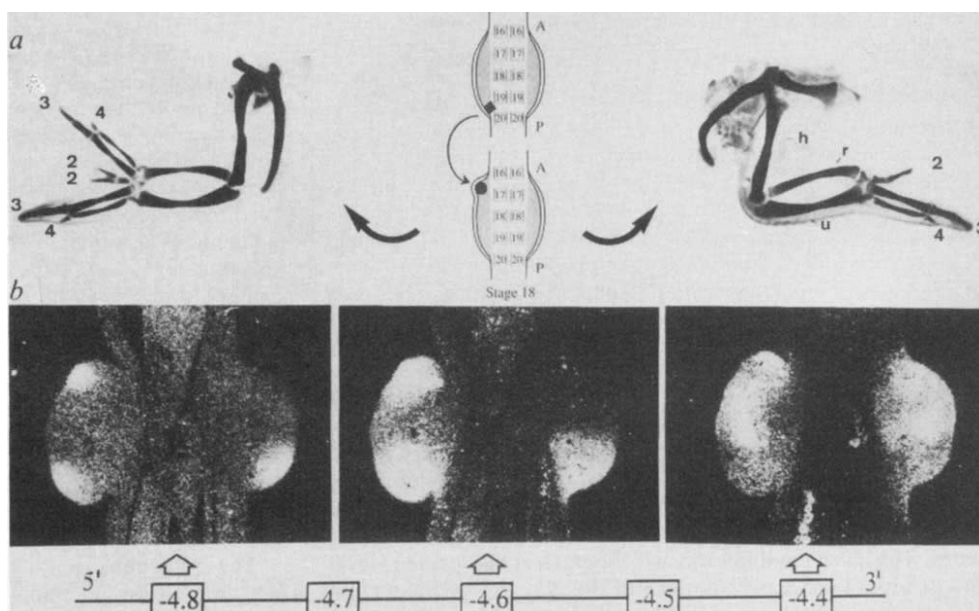
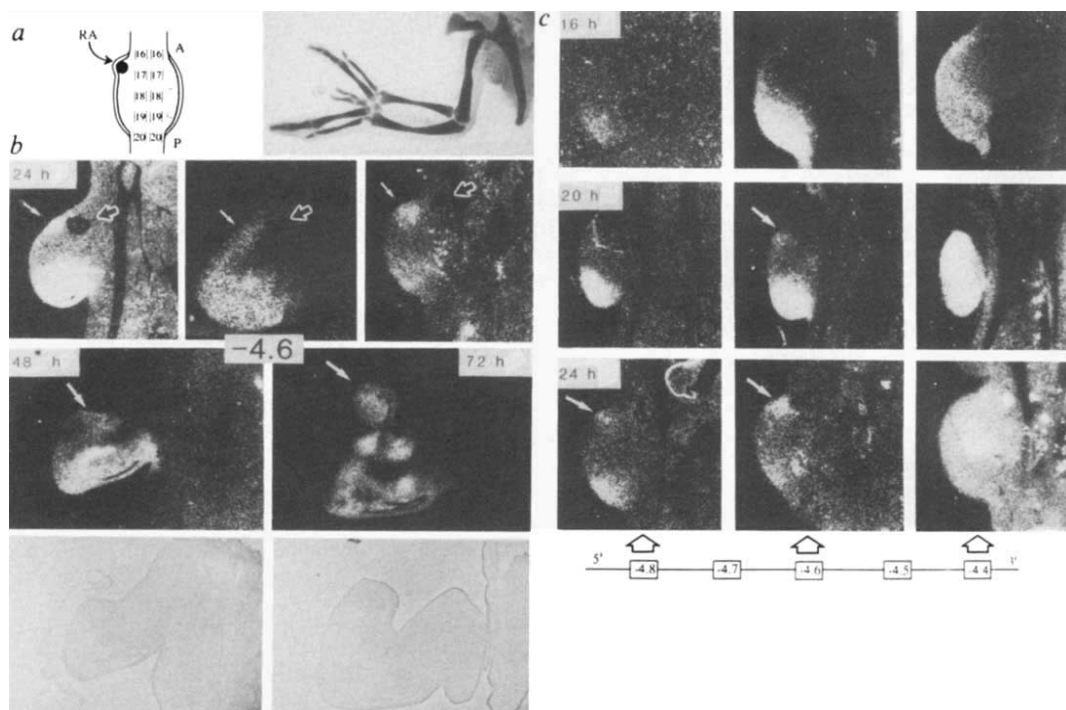


FIG. 4 *Hox-4* gene expression domains in wing buds treated with retinoic acid. **a**, Scheme of the experiment (left) and wing pattern that results (right). A bead soaked in retinoic acid (black dot) was introduced at the anterior margin of the wing bud. An example of the mirror-image digit pattern usually obtained by such a manipulation is shown. Digit pattern is the same as in Fig. 3a. **b**, Expression of the *Hox-4.6* gene in treated wings after 24 h (top), 24-h treatment plus 24-h culture (48* h, middle left) or at 72 h of treatment (middle right) and corresponding bright-field views (bottom). Three independent examples show the *de novo* transcription of the *Hox-4.6* gene in the anterior parts (white arrows) of the wings, next to the holes left by the removal of the beads (open arrows), after 24 h of retinoic acid treatment. The white arrows in the bottom panels point to the extension of these *Hox-4.6* ectopic domains after 40 or 72 h. **c**, Appearance of the *Hox-4* ectopic expression domains in wing buds treated with retinoic acid for 16 (top), 20 (middle) or 24 (bottom) hours. The three genes used are shown at the bottom. The white arrows show that the *Hox-4.6* anterior domain is not detected before 20 h of treatment and appears clearly before that of *Hox-4.8* (absent at 20 h but weakly appearing at 24 h). In this series of experiments, 7 out of 10 treated wings that were allowed to develop for 7 days had digit patterns containing an



extra digit 4 (432234, 43234) and 3 had the pattern 32234. A, anterior; P, posterior.

domains (Fig. 5c,d). This contrasts with the pattern of expression in buds after treatment with 0.1 mg ml^{-1} retinoic acid for a similar time period (48 h), in which there is clearly an anterior ectopic region of expression separated from the normal posterior region of expression by a central region of cells that express only the 3' members of the HOX-4 complex (Fig. 5c,d). At 72 h the entire tip of the bud treated with a high dose expresses *Hox-4.8*, except in regions where cartilage has developed (Fig. 5d). The failure of development of digits 2s in wing pattern is correlated with the absence of a region of cells at the tip of the bud that express only 3' members of the HOX-4 complex, such as *Hox-4.4*.

A molecular view of limb patterning

Our results are consistent with the idea that expression of the *Hox-4* genes is related to the specification of cell position in the limb. In wing buds that will develop mirror-image patterns of digits, there are striking mirror-image patterns of *Hox-4* gene expression. The correct proximodistal sequence of structures in the extra digits may be related to the proper timing in the onset of the *Hox-4* ectopic transcript domains. A comparison between the patterns of digits and of gene expression suggests that the formation of particular digits depends on which *Hox-4* genes are expressed. Cells that will give rise to the posterior digit express all *Hox-4* genes, whereas cells that give rise to the anterior digit do not express the most upstream members of the complex. But digit patterns 434 and 4334 develop with an apparently uniform distribution of *Hox-4.8* transcripts across the tip of the limb, suggesting that both digits 3 and 4 arise from cells that express the same qualitative combination of *Hox-4* genes. The *Hox* combination will operate at the protein level and the quantitative distributions of the *Hox-4* proteins remain to be determined.

extra digit 4 (432234, 43234) and 3 had the pattern 32234. A, anterior; P, posterior.

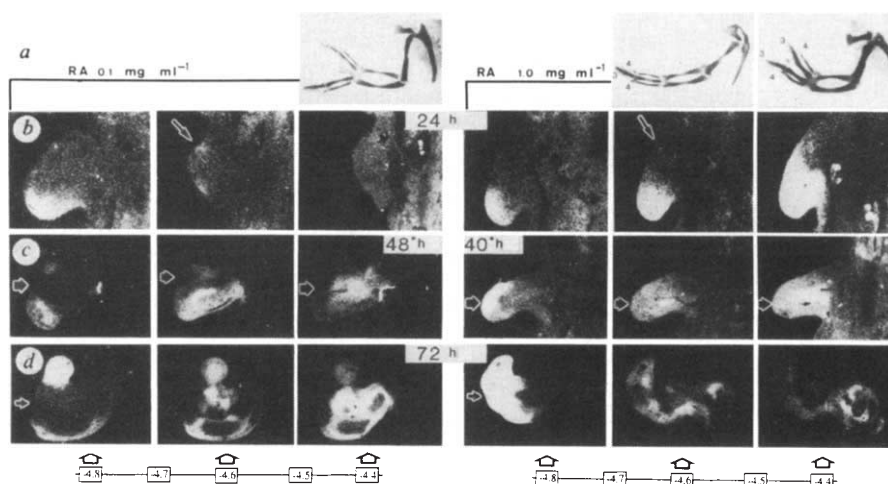
METHODS. AG1-X2 beads (200 μm in diameter) were soaked in a solution of 0.1 mg ml^{-1} all-*trans*-retinoic acid (Sigma; batch number 98F-0778) in dimethylsulphoxide, rinsed and implanted at the anterior margin of chick wing buds (stage 18) beneath the apical ectodermal ridge (for details, see ref. 17). The beads were removed from the wing bud just before fixing except for asterisked time point(s) when they were removed at 24 h and the embryos then reincubated.

The strict temporal sequence of expression of the *Hox-4* genes in normal buds is re-set in anterior cells while they are being reprogrammed to form posterior structures. The rule seems to be that cells cannot express upstream members without first expressing downstream members. This may be important in the ordering of digits and may account for the fact that a digit 2 never develops next to a digit 4. We propose that the incomplete digit duplication patterns obtained with a short duration of retinoic acid treatment (or low dose) are due to the incomplete *de novo* activation of the homoeogenes at the time when patterns are fixed. The length of exposure to retinoic acid that is required for the activation of these posterior genes suggests that retinoic acid may not act directly.

The ectopic expression domains of *Hox-4* genes suggest that each member of the complex may respond to different concentrations of retinoic acid, with genes at the 3' end of the complex being more sensitive than genes at the 5' end. In teratocarcinoma cells, *Hox* genes show this sequence of both sensitivity to retinoic acid and delays in their responses¹⁹. Alternatively, all the genes in the complex could have the same threshold and the spatial pattern would then be determined both by the time that they are turned on and by interactions between them. These ectopic domains induced by retinoic acid treatment closely mirror the normal posterior pattern. This is consistent with endogenous retinoic acid specifying *Hox-4* expression. The localization of the ectopic domains in the progress zone suggests that the pattern of *Hox-4* gene expression must be affected by other factors, such as a graded signal from the apical ectodermal ridge which is required for bud outgrowth^{20,21} and maintenance of the progress zone.

The homeobox genes have a comparable spatial pattern to the limb along the craniocaudal axis during development^{22,23}. This uniformity between the various body axes reinforces the

FIG. 5 Effect of different doses of retinoic acid on the *Hox-4* gene expression domains. Expression domains of the *Hox-4.8*, *-4.6* and *-4.4* genes (bottom line), 24, 48 and 72 h after implantation of beads soaked in 0.1 mg ml^{-1} or 1.0 mg ml^{-1} retinoic acid. The doses used as well as the resultant skeletal patterns are shown in *a*. *b*, Expression domains of the three genes 24 h after implantation of a bead soaked in either 0.1 mg ml^{-1} (left panels) or 1.0 mg ml^{-1} (right panels) retinoic acid. The arrows show the delayed appearance, in the right panels, of the ectopic *Hox-4.6* domain. *c*, Expression domains of the same genes 48 h (left panels) and 40 h (right panels) after treatment with retinoic acid. The asterisks mean that the buds were treated for 24 h and further cultured for 24 or 16 h. Arrows point to the presence or absence of cells not expressing *Hox-4.6* and *-4.8* which would be the new central 'anterior' domain (see the text). *d*, Expression



domains of the same genes after 72 h treatment with retinoic acid. Arrows are as for *c*. Methods as described for Fig. 4.

idea that homeoproteins are involved in early patterning events. Our results support the idea that this gene family could be used for the translation of a primary morphogenetic signal into a complex network of positional information. The different out-

comes of the same system for encoding position arise as a result of the interpretation which will depend on the developmental history of the cells. □

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LETTERS TO NATURE

A rapid energetic X-ray flare in the quasar PKS0558–504

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QUASARS normally exhibit only small fluctuations in brightness, but occasional instances of substantial variability have been used to constrain the size and geometry of the emitting region. Here we report a recent observation from the Ginga satellite¹ of the quasar PKS0558–504, during which the X-ray flux increased by 67% in the space of only 3 minutes. There was no significant change in the spectrum. Comprehensive analysis of the data strongly indicates that this was a genuine X-ray flare originating in the quasar. The implied rate of change in luminosity in the 2–10 keV range, assuming a Hubble constant of $70 \text{ km s}^{-1} \text{ Mpc}^{-1}$ and a cosmological deceleration parameter $q_0 = 0.5$, is $3.2 \times$

$10^{42} \text{ erg s}^{-2}$, the highest value measured for a quasar. When photon scattering is considered, this is ~ 16 times greater than could be produced, with a 3-minute rise time, in an isotropically emitting plasma. We argue that the apparent luminosity must be enhanced by relativistic beaming. This is the first indication of beaming in an 'ordinary' unpolarized quasar.

The quasar PKS0558–504 ($z = 0.137$, $V = 15.0$) was identified optically on the basis of X-ray positions from the HEAO-1 survey². The X-ray spectrum is remarkably steep, with an energy index $\alpha_x = 1.31$ ($S_x \propto E^{-\alpha_x}$, where S_x is the radiation flux per energy interval, and E is the photon energy) of 0.2–7.0 keV. Ultraviolet observations, however, show a flat spectrum, with $\alpha_{uv} \sim 0.5$ (ref. 3). Together, the optical, ultraviolet and X-ray spectra exhibit a very strong 'blue bump' which is currently interpreted as thermal emission from an accretion disk around the central black hole⁴.

Our observations were carried out on 13–14 November 1989 (UT) with the Ginga large area counter (LAC) detectors⁵. Figure 1 shows the X-ray light curve of PKS0558–504 (1–11 keV), plotted in 128-s time bins. The quasar's quiescent emission is characterized here as 'level A' ($6.83 \pm 0.11 \text{ c s}^{-1}$) before 02:30 UT and 'level B' ($5.41 \pm 0.11 \text{ c s}^{-1}$) during a period of lower flux thereafter, although in fact the change may have been gradual. The 2–10 keV luminosity for level A is $6.9 \times 10^{44} \text{ erg s}^{-1}$, which is $\sim 40\%$ of the value of previous measurements^{2,3}.

The flare is shown with increased time resolution (64-s bins) in Fig. 2. The first seven data points have a mean value of $7.73 \pm 0.30 \text{ c s}^{-1}$; we treat this elevated rate (compared with levels A or B) as the baseline flux from which the flare arises. After 01:09 UT, the intensity began to increase reaching a peak $12.90 \pm 0.78 \text{ c s}^{-1}$ at 01:12. The statistical significance of the peak bin alone is 6.2σ , and the integrated flare is more than 8σ above the pre-flare mean. The flare appeared in each of the eight LAC detectors. The post-flare data gap is caused by the reduction of high detector voltages during the readout of the star tracking cameras (a routine procedure).

We have assessed the reliability of the flare by examining possible contaminations. The satellite was in darkness during the flare, excluding any direct detection of solar X-rays. As the quasar was more than 30° above the Earth's horizon, the scattering of solar X-rays by the Earth's atmosphere is also excluded. The LAC field of view was directed $\sim 155^\circ$ from the magnetic field direction, well removed from the 90° direction which can yield soft particle events⁶. The LAC field of view (at 580 km altitude) did not intercept any portions of the South Atlantic

Anomaly or the auroral zones of the south magnetic pole. The IMP-8 satellite did record an increase in the density of the solar wind at a distance of 30 Earth radii before the flare, but monitors of geomagnetic activity and particle density within the Earth's magnetosphere (which includes Ginga's orbit) show no unusual activity at the time of the X-ray event^{7,8}.

The All Sky Monitor and the Gamma Ray Burst Detector have much larger fields of view than the LAC (which covers $1^\circ \times 2^\circ$ full width half maximum), and their background rates seem normal during the flare observations. Data from two other background monitors ('SUD' and 'PIM') are shown in the lower panels of Fig. 1. The SUD rate tracks the background; it consists of LAC detections in the higher energy channels ($>37 \text{ keV}$), where the quasar is well below threshold. If the flare were associated with background events, we would expect a corresponding SUD increase of $\sim 6 \text{ c s}^{-1}$ above the 'normal' rate, which includes a component that fluctuates with the satellite orbit. There is no evidence of such an increase (Fig. 1b). Complementary evidence of background stability is obtained from PIM (Fig. 1c), a shielded anode which records the non-X-ray background. The flare would have raised the PIM rate by $\sim 1 \text{ c s}^{-1}$ if the event were related to the non-X-ray background.

In 210 Ginga blank-sky LAC observations, selected and background-subtracted with the same criteria and methods as in the current data, no events that resembled the intensity and short timescale of the flare were found. A further test for X-ray detections is to compare the count rates of two layers of the LAC instrument. X-rays below 10 keV are mostly absorbed (and detected) in the top layer, but a fraction will penetrate to the middle layer. During the flare, the count rates in the middle layer did show a modest increase (4.1σ), of the amplitude expected for an X-ray source with the spectral shape of the quasar. We conclude that all the available diagnostics indicate that the event is a genuine X-ray flare.

Finally, could another X-ray source in the LAC field of view have emitted the flare? Stars with active coronae are known to flare in X-rays, on timescales from minutes (typically with 10–30% flickering) to hours for dMe stars⁹, and from hours to days for RS CVn stars¹⁰. But the EXOSAT low-energy instrument (LE) image of PKS0558–504 shows no other X-ray detection (0.2–2.0 keV) within $\sim 1^\circ$ of PKS0558–504. In addition, the flare spectrum (Fig. 3 and text below) is very similar to the quasar's quiescent spectrum, with an equivalent temperature higher ($T > 6.3 \text{ keV}$ to 90% confidence) than would be expected from dMe stars^{11,12}. Several fast X-ray transients observed with the HEAO A-2 survey¹³ are largely unidentified, but there is one case

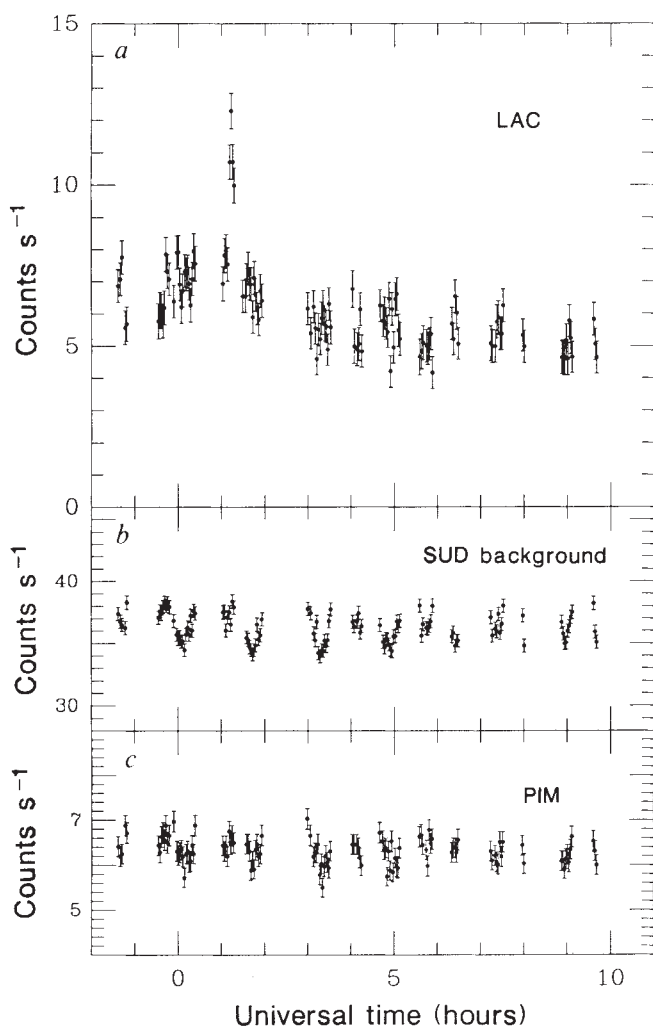


FIG. 1 (a), Net counting rate (1–11 keV) of PKS0558–504 in the top layer of the Ginga LAC detector. b, Count rate in the upper level discriminator (SUD), which tracks the total background rate. c, Counting rate for the PI Monitor (PIM), which is correlated with the rate of non-X-ray background events. The gaps in the data are due primarily to earth occultations, high background regions and readouts of the star tracker. The data have been corrected for spacecraft attitude and selected to eliminate times of high background and possible particle contamination⁶. The background rate ($\sim 26 \text{ c s}^{-1}$) has been subtracted; both methods described in ref. 6 yield similar results.

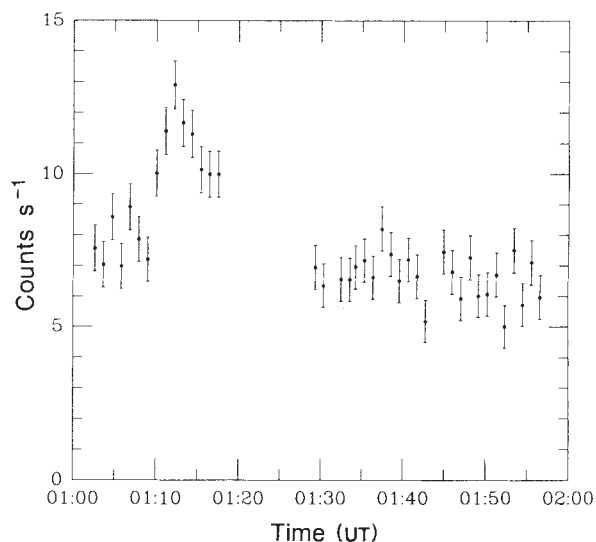
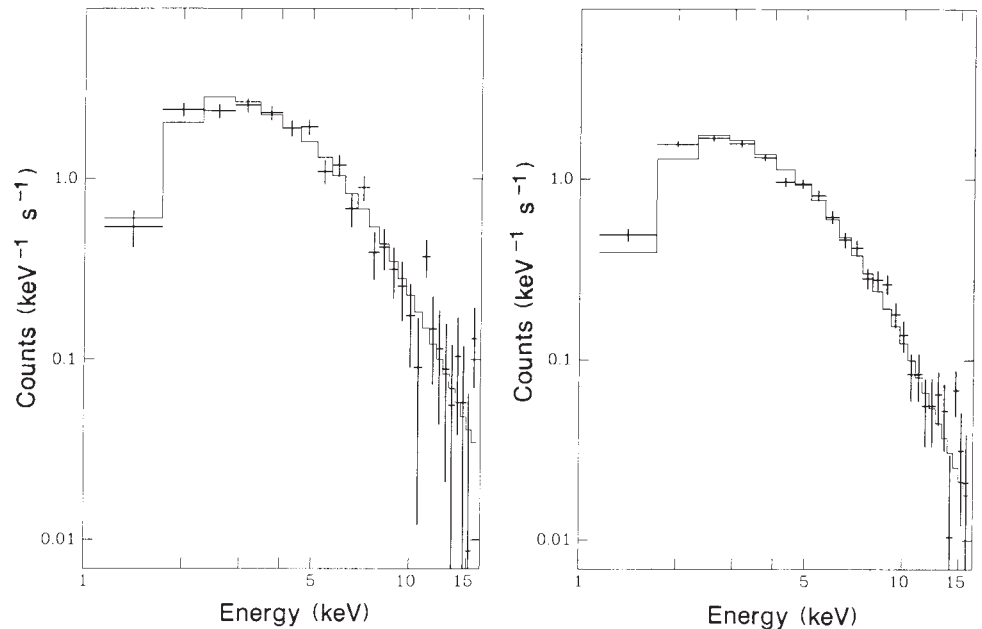


FIG. 2 Same as Fig. 1a, but with an expanded horizontal scale and 64-s time resolution.

FIG. 3 *a*, Spectrum of PKS0558–504 during the central 360 s of the flare event. *b*, Spectrum during level A. The solid lines show the spectral fit for a power-law model, with the spectral parameters listed in Table 1.



(H0449–55) in which a confirmed dMe flare reaches a temperature of 10–17 keV. The flare timescale, however, is 3 hours¹⁴, and soft X-ray emission is detected in quiescence (EXOSAT LE database). The quasar is therefore the likely origin of the Ginga flare.

X-ray spectral fits were computed for the three observation segments: level A (excluding the flare), the flare interval and level B. The Ginga observations do not yield a significant intervening column density, and there is evidence of soft X-ray excess at energies ≤ 2 keV. Therefore, we assume a fixed value, $N_H = 10^{20.6} \text{ cm}^{-2}$, derived from the observation with EXOSAT³ which is sensitive to absorption effects at low X-ray energies. All the power-law fits (Table 1) are roughly consistent with the mean index $\alpha_x \approx 1.1$. We also model the net flaring spectrum (flare interval minus level A); a power-law model applied to this spectrum yields an energy index that is consistent with the shape of active galactic nuclei (AGN), of which quasars are a subclass. This is also a powerful discriminator against particles entering the front window, as such events yield $\alpha_x < 0$.

This flare is one of the most dramatic examples of rapid variability from an AGN. Recently, the quasar 3C279 ($z = 0.538$) exhibited a 20% flux increase in 45 minutes (ref. 15) achieving a change in luminosity (2–10 keV), $dL/dt > 2.9 \times 10^{42} \text{ erg s}^{-2}$. The X-ray rise of PKS0558–504 implies $dL/dt \approx$

$3.2 \times 10^{42} \text{ erg s}^{-2}$. Both results represent a marked departure from the behaviour of emission-line AGN, reaching the variability levels previously attained only by BL Lac nuclei^{16,17}. One BL Lac object, H0323+022, exhibited a 30-s X-ray dip¹⁸ which yields $dL/dt \approx 5 \times 10^{43} \text{ erg s}^{-2}$.

In the interpretation of AGN variability timescales^{17,19,20} an ‘efficiency limit’ (η) is derived for the rate of conversion of gravitational potential energy into X-ray emission, assuming that the photons must escape through the accreting matter, and that the photon opacity is dominated by Thomson scattering. The derived limit is $dL/dt < 2 \times 10^{42} \eta \text{ erg s}^{-2}$. It is unlikely that η exceeds 0.1; for PKS0558–504, the observations imply $\eta \approx 2$, which is clearly impossible.

The breakdown of nonrelativistic assumptions can also be described as follows. The flaring timescale (Δt) provides, from the causality argument, an upper limit for the size of the emitting region $R < c\Delta t$. In the case of PKS0558–504, $R < 5.4 \times 10^{12} \text{ cm}$ (0.36 AU). If the average luminosity is within the Eddington limit (gravity overcomes radiation pressure; $L_{\text{Edd}} = 4\pi GMm_p c / \sigma_T$), then the quasar must have a central mass $M > 10^8 M_\odot$. But the Schwarzschild radius for such a black hole would be larger than the emission region ($R_s = 2GM/c^2 > 3 \times 10^{13} \text{ cm} > R$), so no emission should escape.

Such contradictions have been used to argue for relativistic beaming in BL Lac nuclei, but at present PKS0558–504, 3C279 and possibly LB9743 (ref. 21) are the only examples among quasars. The steep X-ray index of PKS0558–504 resembles BL Lac objects, but PKS0558–504 exhibits characteristics of quasars, including strong emission lines², an impressive ‘blue bump’³, a lack of optical polarization²² and a decreasing radio spectrum (energy index $= 0.53 \pm 0.07$; ref. 22). Whereas 3C279 belongs to a subclass known as ‘optically violent variables’, optical photometry^{2,22} of PKS0558–504 at 6 epochs from 1985 until the first day of the Ginga observations is consistent with the mean value $V = 15.05 \pm 0.05$. Evidence of anisotropy in ‘normal’ quasars suggests that the total number may be larger than the population we observe (those beamed along our line of sight).

Further evidence for beaming in AGN is the observation of superluminal motion in the cores of radio sources²³. The other quasar with large X-ray variations, 3C279 (ref. 24), also exhibits superluminal motion, as does the BL Lac object OJ 287 (ref. 25). X-ray and radio observations of beaming phenomena may provide independent views of the relativistic jets, as the kinematic Doppler factor and the physical location of the emitting region

TABLE 1 Model fits* and spectral parameters

	Energy index α_x	Constant†	χ^2_ν	X-ray flux‡
Level A (excluding flare)	1.01 ± 0.07	6.2 ± 0.6	1.73	1.57×10^{-11}
Flare interval§	0.92 ± 0.12	9.0 ± 1.5	1.25	2.64×10^{-11}
Level B state	1.24 ± 0.08	6.2 ± 0.6	1.97	1.11×10^{-11}
Net flare	0.75 (+0.31, –0.27)	2.9 ± 1.3	0.93	
Net flare (thermal bremsstrahlung)	$kT = 10.8$ (+9.3, –4.5)	2.1 ± 0.42	0.90	

* All error bars are 90% confidence limits, varying one parameter at a time, with 29 degrees of freedom for the first three fits and 14 for the net flare spectrum. The χ^2_ν values for level A and B are inflated by soft X-ray excess at energies < 2 keV.

† Units are $10^{-3} \text{ photons cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1}$.

‡ X-ray flux at 2–10 keV, in units of $\text{erg cm}^{-2} \text{ s}^{-1}$.

§ The flare interval consists of 360 s of data centred on the peak flux.

|| Flare minus level A

may change with photon energy²⁶. We know of no high-resolution radio observations of PKS0558–504.

The physical origin of the PKS0558–504 flare remains unknown, but two broad possibilities are (1) an abrupt change in the travel path of a relativistic jet, causing the radiation beam to sweep across the line of sight, or (2) the birth, acceleration and rapid decay of a relativistic blob which is beamed at us and exhibits evolutionary timescales markedly reduced by a large Doppler factor. As the observations of the flare are confined to the X-ray region, we urge multi-frequency, ground-based support during future high-energy observation of this quasar. □

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A γ -ray burst preceded by X-ray activity

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GAMMA-RAY bursts remain mysterious astrophysical phenomena. The discovery of cyclotron harmonics^{1–3} in their spectra is strong evidence that they originate from strongly magnetized neutron stars, but their energy source and photon production mechanism are unknown. In observations of a γ -ray burst using the Ginga astronomy satellite, we detected X-ray emission in the 2–10 keV energy range ~ 10 s before the onset of the γ -ray event, as well as a tail of X-ray emission for ~ 30 s afterwards. The long timescale and near black-body spectrum of the precursory X-ray emission suggests that the burst mechanism involves a transition from thermal to non-thermal photon production, and that the energy source for the burst comes from within the neutron star rather than from accretion.

The first γ -ray burst observed in the X-ray photon energy band was GB720427, which was detected in the 2–8 keV range⁴. X-ray data from GB720514 provided some evidence⁵ that an accompanying X-ray signal might have lasted longer than the γ -ray event. Detailed X-ray observations of four catalogued γ -ray bursts obtained with the *P78-1* satellite⁶ showed that, in general, there is no detailed correspondence between the X-ray and γ -ray signals. Of particular interest is the evidence for X-ray precursor activity in three out of the four events, and the observation of an X-ray tail in one of them. Two X-ray events⁷ observed with the *Hakucho* satellite were also coincident with γ -ray bursts. One of these, GB811016, had an X-ray tail which lasted for ~ 30 s after the γ -ray event had terminated.

To follow up these results, a γ -ray detector with low-energy X-ray capability was designed to be launched on the *Ginga* satellite⁸. This instrument consists of a proportional counter (PC) covering the 1–30 keV range, and a scintillation counter (SC) which is sensitive to photons of 15–400 keV. Each counter has an effective area⁹ of ~ 60 cm².

Seventeen intense events detected by *Ginga* had good signal-to-noise ratios, and eight showed a clear soft X-ray tail after the termination of the γ -ray photons^{10,11}, lasting for up to ~ 100 s. During some of these tails the X-ray intensity rose to a second maximum. Such recurring X-ray peaks had been observed previously⁷. The event reported here was detected on 26 January 1990 18.04.23 UT by *Ginga* and by the *Watch* experiment on board the *Granat* mission. In Fig. 1 we show the profiles of the burst as observed in the PC and SC counters. The γ -ray signal shows two distinct peaks, separated by ~ 6 s, both of which have rise times of ~ 1.5 s. A particularly striking feature of the burst profile is that the start of the X-ray signal occurred ~ 10 s before the onset of the first peak of the γ -ray burst. This type of initial phase X-ray signal was observed in only this case out of 17 intense events. The dead-time-corrected X-ray and γ -ray signals reached maximum values of $\sim 4,500$ and $\sim 2,250$ counts s^{−1} above the background, respectively. Using the parameters of the spectral model that fits the observed spectrum, these peak count rates correspond to peak fluxes of 7×10^{-7} and 1.3×10^{-5} erg cm^{−2} s^{−1}, over the energy intervals 2–30 keV and 15–400 keV, respectively. The γ -ray burst lasted for ~ 15 s after the onset, and it had a fluence of $\sim 4 \times 10^{-5}$ erg cm^{−2} in the range 1.5–400 keV. These peak fluxes and the fluence have not been corrected for the aspect of the detector.

We have made spectral fits to the combined PC and SC data for a number of 0.5-s time intervals near the peaks of the burst. As a measure of the background we took the data obtained during the first 8 time bins in the data record (see Fig. 1). We found that the spectral shape at the first peak of the γ -ray burst can be modelled as a thermal-bremsstrahlung spectrum with a temperature of ~ 120 keV. At the second peak the thermal-bremsstrahlung temperature was ~ 85 keV. These peak temperatures and the softening have frequently been observed^{10,12} in classical γ -ray bursts in the energy region below ~ 500 keV. We have made fits to the spectrum observed with the PC during the 8-s time interval before the onset of the γ event. We used power-law, thermal-bremsstrahlung and black-body spectral models. We find that this initial phase spectrum can be reasonably well described by that of a black body with a temperature T given by $kT = 1.58 \pm 0.26, -0.23$ keV (90% confidence levels) as shown in Fig. 2. The reduced χ^2 of this fit is 1.6 for 12 degrees of freedom. The power-law and thermal-bremsstrahlung fits are substantially worse, with reduced χ^2 values of 3.6 and 2.1, respectively. There was also a faint signal in the SC, but this was very soft, having the character of an X-ray tail to the black-body radiation rather than being a hard spectrum from an underlying classic γ -ray burst. The average X-ray flux in the initial phase was $\sim 2.5 \times 10^{-9}$ erg cm^{−2} s^{−1}, which, if we assume spherical emission of the signal and a temperature of 1.58 keV, corresponds to an apparent black body radius $r \sim 0.6$ ($d/1$ kpc) km. Here d is the source distance.

If we assume that the spectrum in the initial phase is that of a black-body (or black-body-like) emitter, the increase of the signal could be caused by a change either in the projected area of the emitter or in its temperature. We have attempted to test these two possibilities. Because statistical errors on the background-subtracted count rates in the individual 0.5-s time bins were large, we divided the initial phase into just two parts, with roughly equal numbers of total counts. Similarly, we divided the photon energy range of the PC counter into two channels, 1–4 keV and 4–7 keV, with backgrounds of 78.6 ± 3.8 counts per 0.5 s and 82.7 ± 2.2 counts per 0.5 s respectively (errors are 1σ).

To test the hypothesis that the black-body temperature of the emitter is constant, but its area is changing, we can simply test for the constancy of the hardness ratio, R , of the counts in these two channels. We find $R = 1.56 \pm 0.7$ and 1.33 ± 0.36 for the first and second part of the initial phase respectively. Thus the statistics do not allow us to decide whether the temperature is constant. To test the alternative hypothesis that the area of the emitter is constant, but the temperature is increasing, we searched for possible temperature variations that reproduce both the observed variation of the total count rate in the initial phase, and the observed hardness ratios in the two broad time bins. We used a relation between black-body temperature and total count rate obtained by folding Planck functions with the detector response to transform the observed count rate variation (for each 0.5-s time bin) into a temperature variation. With these temperatures, we then determined the count rates expected in each of the two broad spectral channels. By adding these counts over the two parts of the initial phase separately for the two

spectral channels, we obtained two predicted values of the hardness ratios. As the observations cannot determine the normalization of the black-body model, we had to fix the temperature at one point; we chose the end of the initial phase, when the signal and, therefore, the temperature are maximal. In view of the black-body temperature obtained from the spectral fit to the total initial-phase spectrum, we took values for the trial temperature ranging between 1.0 and 3.0 keV. For values of this maximum black-body temperature in the range 2.1 to 2.5 keV, our analysis showed that the second hypothesis could account for the observed variations in hardness ratio and total counting rate, but the statistical quality of the data during the initial X-ray phase is insufficient to allow us to distinguish between changes in temperature and changes in area of the assumed black-body emitter as the cause of the X-ray flux increase.

There is strong evidence that γ -ray bursts originate from strongly magnetized neutron stars, both from observation^{1–3} and from theoretical arguments^{13–15}. Models can be divided into two groups according to the energy source assumed for the γ -ray bursts: (1), the γ -ray bursts are caused by sudden accretion events, and their energy source is gravitational^{16,17}; (2), the energy originates from within the neutron stars, for example, as thermonuclear or rotational energy, or stresses in the neutron star crust^{18,19}. A prerequisite for γ -ray emission would seem to be the absence of thermalization of the photons generated near the neutron star surface. The observed rise time of ~ 8 s in the X-ray initial phase is longer than the dynamical timescale expected for the accretion of solid bodies such as comets¹⁷. Therefore, if the γ -ray burst was an accretion event it seems likely that the accreting matter was stored, for example, in an accretion disk²⁰. In that case the rise time of the event may reflect the time over

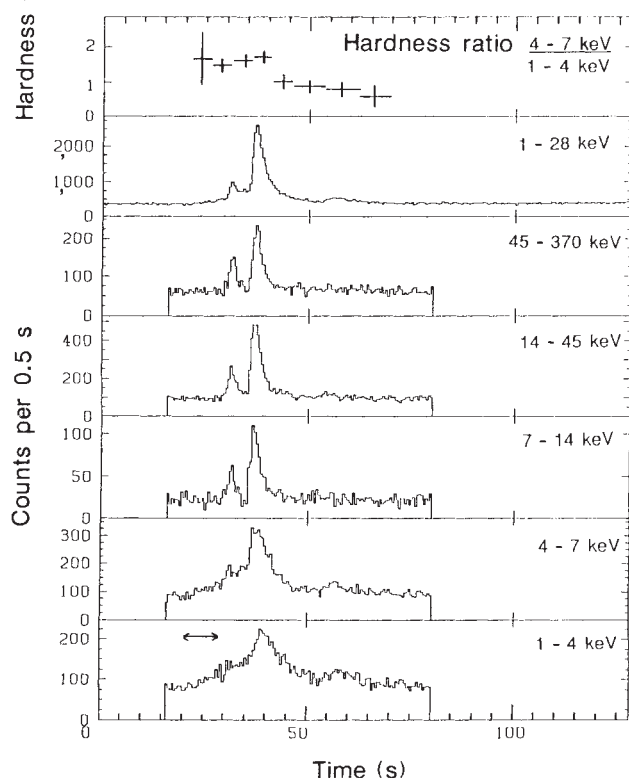


FIG. 1 Variation of intensity and hardness ratio in the γ -ray burst GB900126. The uppermost panel shows the hardness ratio of the number of counts in the energy channels 4–7 keV and 1–4 keV, after background subtraction. The second panel from the top, which covers a time interval of 32 s before and 96 s after the first peak, shows a time profile of GB900126 in the energy interval 1–28 keV after dead-time correction. The remaining five panels contain the burst profiles in the energy ranges 1–4 keV, 4–7 keV, 7–14 keV, 14–45 keV and 45–370 keV, without dead-time correction, for a time interval of 16 s before and 48 s after the first peak. The arrow in the bottom panel indicates the interval for Fig. 2.

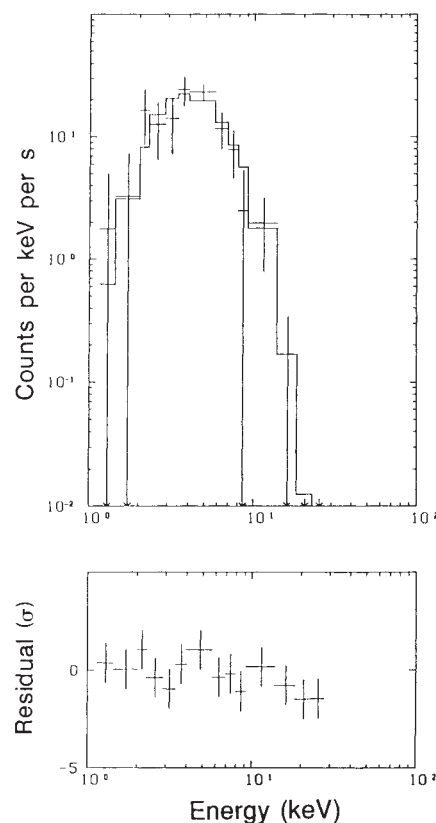


FIG. 2 Raw background-subtracted spectrum during the X-ray initial phase before the first peak (shown by arrow in Fig. 1). The solid line indicates the best fit black-body model, with a temperature of 1.5 keV.

which the accretion instability develops. For all known unstably accreting neutron stars, however, the bulk of the emission is generated as X-rays, independent of the accretion rate or the strength of the neutron star magnetic field.

Our spectral fittings indicate that a possible origin for the X-rays observed in the initial phase to GB900126, and during the tail following this event, is thermal emission from the surface of a neutron star. This can be naturally accounted for by the second group of models above. We cannot, on the basis of our spectral fits, distinguish between temperature variations and area variations as the cause of the X-ray flux increase during the initial phase. However, if we assume that the increase was caused by a temperature increase at a constant area, we find that at the end of the X-ray initial phase, just before the onset of the γ -ray emission, the apparent black-body temperature reached a maximum value kT in the range 2.1–2.5 keV. Although

the Eddington limit will be affected by the strong magnetic field, it is worth noting that this maximum temperature is very similar to the maximum temperature observed during X-ray bursts in which the luminosity reaches the flux level of the local Eddington limit, showing evidence for mass loss from the neutron star surface^{21,22}. Under the above assumption, this might suggest that the onset of the γ -ray event occurs when either the X-ray flux from the neutron star surface or its temperature reaches a critical value, as is required by some γ -ray burst models^{14,19}. The existence of a soft photon source near 1 keV is required by Cyclotron up-scattering models for γ -ray burst spectra^{23,24}. The observation of such a photon source accompanying γ -ray bursts gives some support to these models. But it is unclear how, after the initial emission of low-energy X-rays, indicating that the emission is thermalized, the system can subsequently avoid this thermalization and emit high energy γ rays. $\square$

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Observation of the optical analogue of quantized conductance of a point contact

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DIFFRACTION of light by an aperture is a well-known manifestation of the wave nature of light. The most familiar case is that of an incident plane wave, which is diffracted into a spatial pattern that is sensitive to the properties of the aperture: the ratio of transmitted power to incident flux (the transmission cross-section σ) depends in a complicated way on the aperture area A (refs 1–3). For diffuse (that is, isotropic rather than plane-wave) illumination, however, the situation is much simpler⁴: in three dimensions, σ increases with A in a series of steps of equal height $\lambda^2/2\pi$ (where λ is the wavelength of the light), and is thus independent of the detailed aperture shape. A similar simplification occurs for two-dimensional diffuse illumination of a slit: the transmission cross-section per unit slit length increases in stepwise fashion as a function of the slit width W , with steps of height $\lambda/2$ occurring whenever $W = n\lambda/2$ ($n = 1, 2, 3, \dots$)—that is, whenever a new mode is enabled in the slit. Although the optical transmission characteristics of slits have been studied extensively for plane-wave illumination^{5–8}, we know of no investigation of this predicted staircase dependence for diffuse illumination. Here we report the observation of such an effect, and suggest that it may play a part in any process of wave propagation through a constriction.

The argument of ref. 4 is based on the analogy with the recently discovered quantization of conductance in ballistic

electron transport^{9,10}. The conductance of a point contact in a two-dimensional electron gas increases in steps of $2e^2/h$ as its width is increased (e is the charge on an electron). The origin of this effect is the quantization of the electrons' transverse momentum owing to lateral confinement within the point contact. This leads to the formation of one-dimensional sub-bands in the conduction band (analogous to the formation of transverse

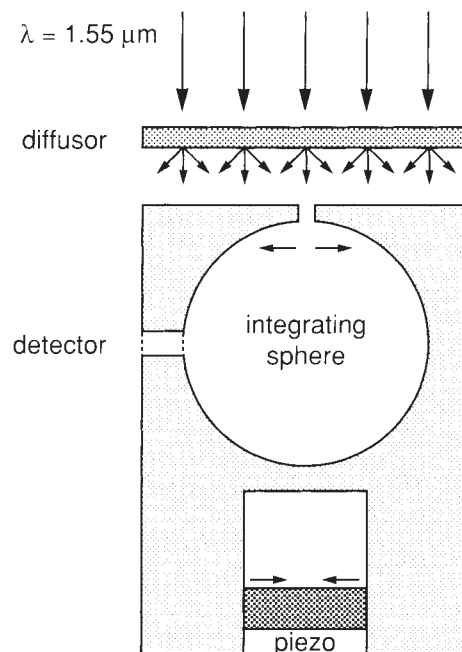
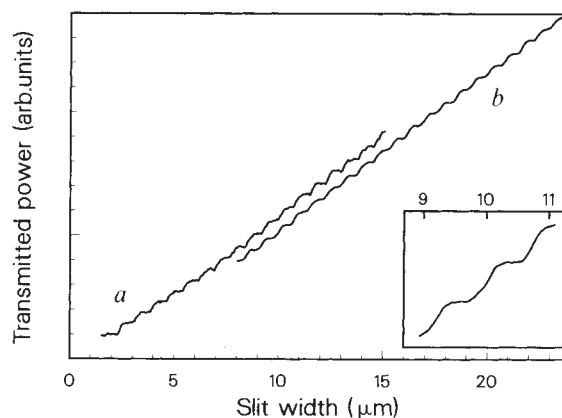


FIG. 1 Schematic illustration of the apparatus.

FIG. 2 Transmitted power as a function of the slit width, using a paper diffuser (a) and a glass-fibre diffuser (b); trace b is scaled and shifted vertically for clarity. The inset shows an enlarged part of trace b.



modes in a waveguide), each occupied sub-band contributing $2e^2/h$ to the conductance. Planck's constant h appears in the step height as a result of the application of Fermi statistics to a degenerate electron gas. Obviously, this consideration does not carry over to the optical case. But the staircase dependence of conductance on the width of the constriction is not directly related to the fermionic nature of electrons, and has its optical analogue in the predicted stepwise increase of the transmission cross-section of a diffusely illuminated slit⁴. The diffuse illumination is analogous to the isotropic velocity distribution of the incoherent electron waves incident on a point contact.

We performed our experiments at a wavelength of $1.55\text{ }\mu\text{m}$. This value of λ is sufficiently large to allow fabrication of a slit with tolerances of $\lambda/10$, while enabling the use of a semiconductor laser and a sensitive Ge diode detector. The set-up is shown schematically in Fig. 1. The body of the device consists of two halves of an integrating sphere (40 mm diameter) made of aluminium and coated with diffusely scattering barium sulphate. The slit is situated in a region of the sphere at which the metal is only $25\text{ }\mu\text{m}$ thick. The length of the slit is $200\text{ }\mu\text{m}$. Inside the slit, the aluminium is covered with silver to obtain a high reflection coefficient (0.98). This, and a small thickness, are required to avoid destruction of the transmission staircase by excessive absorption at the walls of the slit. The radiation transmitted through the slit in all directions is collected by the integrating sphere and detected by a Ge diode. The slit width is varied by separating the two halves of the sphere using a piezo-electric transducer, which allows a $15\text{-}\mu\text{m}$ scan in width (added to a manual offset). The variation of the slit width is monitored by a Michelson interferometer operating at 633 nm , with one of the mirrors attached to the device.

The laser beam, modulated at 1 kHz and phase-locked detection, is expanded by a microscope objective lens and scattered by a diffuser. Diffuse illumination in two dimensions only (no propagation in the direction parallel to the slit) is crucial, because we use a slit rather than an aperture. We obtained two-dimensional diffuse illumination in two different ways. In the first, a piece of white plotting paper was used as a three-dimensional diffuser. To eliminate divergence parallel to the slit, two narrow slit-shaped diaphragms were placed between the diffuser and the slit. This resulted in an unpolarized, two-dimensionally diffuse light source. Alternatively a random array of parallel glass fibres was used as an intrinsically two-dimensional diffuser¹¹, giving polarized diffuse illumination with the electric field parallel to the slit. In both methods, two-dimensionally diffuse illumination of the slit was achieved in an opening angle from 0° to 85° normal to the slit. The construction of our device did not allow illumination from -85° to 0° , nor illumination at grazing angles (in excess of 85°). Neither of these omissions affects the results. Because of the large bandwidth of the laser (15 nm), the illumination is essentially incoherent.

The experimental results are presented in Fig. 2, which shows the transmitted power as a function of the slit width. The two curves *a* and *b* show the results obtained using the paper diffuser and the glass-fibre diffuser respectively. The latter method produces a higher illumination intensity and thus a better signal-to-noise ratio. A stepwise increase of the transmitted power is clearly observed in both cases. The steps occur at width intervals of $\lambda/2$, as predicted⁴. The first two steps were not observed, because the slit could not be closed completely for mechanical reasons. The transmitted power is equal to the transmission cross-section per unit length, σ' , multiplied by a normalization factor. For large slit widths ($W/\lambda \rightarrow \infty$), σ' is equal to W ; it therefore follows that the height of the steps in σ' must be equal to the size of the intervals in W , that is, $\lambda/2$.

The steps in the transmission cross-section are not abrupt. This is caused partly by non-uniformities in the slit width, and partly by the slight absorption of radiation at the walls of the slit, which occurs in spite of the use of a silver coating. The resulting damping of the propagating modes¹¹ causes a rounding of the steps and a slight curvature of the staircase for the first few steps, as seen in Fig. 2*a*. Rounding of the steps is also partly due to non-adiabatic coupling (with inter-mode scattering) between the narrow slit and the infinite exterior space¹².

Our choice of the slit geometry was motivated partly by a desire to make the analogy with the two-dimensional electron gas, and partly by experimental considerations: a diaphragm of variable area of the order of λ^2 is rather difficult to fabricate, and in addition the total transmitted power is much smaller than that for a slit. Nevertheless, the extension of the experiment to three-dimensional illumination is of interest because it brings the vector character of light into the problem in a non-trivial way (two-dimensional diffraction being essentially a scalar problem¹).

To conclude, we find it remarkable that this optical phenomenon, with its distinctly nineteenth century flavour, was not discovered before its electronic counterpart. $\square$

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Normal-state conductivity between CuO_2 planes in copper oxide superconductors

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THE high-transition-temperature (high- T_c) copper oxide superconductors are highly anisotropic in their electrical properties; this is one of the key concerns for applications of these materials, and may also provide a clue to the mechanism of the superconductivity. Although the resistivity in the CuO_2 planes, ρ_{ab} , shows a metallic temperature dependence, the resistivity parallel to the c axis (ρ_c) has been reported to be both non-metallic^{1,2} and metallic^{3,4}. Here we present systematic data for ρ_c in a number of high- T_c materials, obtained from well characterized single crystals. Both the magnitude and the temperature dependence of ρ_c are strongly dependent on crystal structure, and on the concentration of charge carriers. We find that ρ_c is non-metallic ($d\rho_c/dT < 0$) in most superconducting compounds, suggesting an unconventional conduction mechanism. Fully oxygenated $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ (with $T_c \approx 90$ K) is the only exception, with a relatively small ρ_c and positive $d\rho_c/dT$ that may arise from the crystal structure specific to this material.

Single crystals were grown by the conventional CuO flux method in a platinum crucible. In the case of $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$, the crystals grew by cooling at the rate of 1°C h^{-1} from $1,000^\circ\text{C}$ to 890°C ; in the case of $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$, at the rate of 5°C h^{-1} from $1,350^\circ\text{C}$ to $1,200^\circ\text{C}$. The strontium concentration of $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ crystals was checked by electron-probe microanalysis which revealed homogeneous distribution of Sr with accuracy better than 0.01 for x . The oxygen content of $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ crystals was controlled by annealing them at 400°C for four days in a sealed quartz tube together with a large amount of $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ powder (~ 10 g) which had a prescribed oxygen content. We annealed at relatively low temperature ($\sim 400^\circ\text{C}$) to minimize the disorder or defects in a crystal, to which ρ_c is found to be sensitive. The annealing period is long enough for oxygen to diffuse deep into crystals, for they show a very sharp superconducting transition in resistivity, and further annealing made no change in transport properties. The oxygen content of annealed single crystals should be identical to that of the powder sealed with them, which was determined by iodometric titration. The resistivity ρ_{ab} of fully oxygenated samples at room temperature is $\sim 150 \mu\Omega \text{ cm}$, which is among the lowest that have been reported and guarantees good quality.

Measurements of the anisotropic resistivity were performed on rectangular single crystals (typically $1.0 \times 1.0 \times 0.05 \text{ mm}^3$) by the Montgomery method⁵. Contacts with diameter $< 0.05 \text{ mm}$ were attached by heat-treatment-type gold paste, as shown in the inset of Fig. 1, at the corners of bottom and top surfaces parallel to the CuO_2 plane to avoid misalignment of electrodes. The temperature dependence and the magnitude of ρ_{ab} measured by the Montgomery method coincide well with those measured by the conventional four-probe method (inset, Fig. 1) for fully oxygenated 90-K $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ ($y \leq 0.07$), so the reliability of the Montgomery method is confirmed.

There arises the question of whether the measured ρ_c contains a contribution from ρ_{ab} owing to imperfect alignment of layers in the crystal, and thus the intrinsic behaviour of ρ_c is screened out. To make this point clear, we compare the temperature dependence of ρ_c for 90-K and 80-K $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ ($y \approx 0.13$) in Fig. 1. These data were taken on the same crystal by annealing under different conditions in a sealed quartz tube. The result

was reversible and reproducible for repeated annealing. In contrast to the metallic temperature dependence of ρ_c of the 90-K material, the 80-K material exhibits non-metallic behaviour with ρ_c larger by a factor of 6. If the measured ρ_c in the 80-K material were equally affected by ρ_{ab} in both cases, metallic temperature dependence should have dominated in the 80-K crystal because ρ_{ab} of the 80-K crystal is similar both in magnitude and temperature dependence to that of the 90-K crystal. We may safely conclude that the observed temperature dependence of ρ_c for fully oxygenated 90-K $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ is intrinsic.

Figures 2 and 3 show the temperature dependences of ρ_c for $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ and $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ with various hole densities. Although the temperature dependence of ρ_{ab} is in most cases metallic, ρ_c exhibits a marked change in magnitude as well as in temperature dependence for both systems with changing hole concentration. For the compounds with low and moderate hole concentrations, $d\rho_c/dT$ is negative at low temperatures. Only for the highest hole concentration, fully oxygenated $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ ($T_c \approx 90$ K) and $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ with $x = 0.34$ (non-superconducting), does the temperature dependence of ρ_c become metallic down to the lowest temperatures in the normal state. In both systems the anisotropy, ρ_c/ρ_{ab} , decreases markedly with doping, from 1,700 ($y \sim 0.18$, $T_c \approx 60$ K) to 70 ($T_c \approx 90$ K) for $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ and from 8,000 ($x = 0.08$) to 100 ($x = 0.34$) for $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$, where the values of ρ_c/ρ_{ab} are those just above T_c for superconductors and at 4.2 K for nonsuperconductors. The trends found in $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ agree with previous work^{4,6}. Kambe *et al.*⁷ measured ρ_c of $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ ($x = 0.14$, $T_c \approx 30$ K) on a single crystal with the longest dimension parallel to the c axis, which was grown by the travelling-solvent/floating-zone method. ρ_c is non-metallic, and its magnitude is intermediate between our $x = 0.08$ and $x = 0.20$ (Fig. 2). This fact also indicates the reliability and systematic variation of the present ρ_c measurement.

We have extended the measurements to the system $\text{Bi}_2\text{Sr}_{1-x}\text{La}_x\text{CuO}_6$ (Bi-2201 , $T_c \approx 20$ K when $x = 0$), where the hole density is reduced with the La composition x , and to electron-doped $\text{Pr}_{2-x}\text{Ce}_x\text{CuO}_{4-y}$ ($x \approx 0.15$). The result for the latter system are different from previous work on

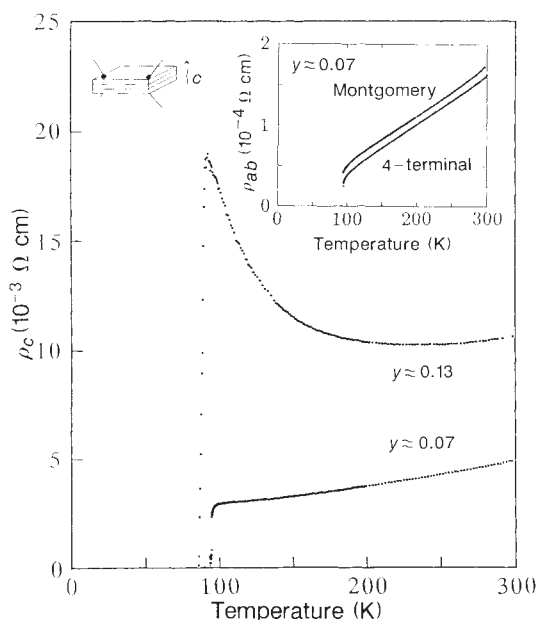


FIG. 1 Temperature dependences for ρ_c of fully oxygenated ($y \leq 0.07$) and lightly reduced ($y \approx 0.13$) $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ prepared by annealing the same single crystal under different conditions. The configuration of the electrodes for measurement by the Montgomery method is shown in an inset. In a second inset the temperature dependence of ρ_{ab} for fully oxygenated $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ measured by the Montgomery method is compared with the conventional four-terminal method.

$\text{Nd}_{2-x}\text{Ce}_x\text{CuO}_{4-y}$ (ref. 8), perhaps because of inhomogeneity in the Ce content which is much more serious in $\text{Nd}_{2-x}\text{Ce}_x\text{CuO}_{4-y}$, but those for the former agree with the recent report by Martin *et al.*⁹ As shown in Fig. 2, ρ_c is very large ($\rho_c/\rho_{ab} \geq 10^4$), and non-metallic ρ_c is observed in both systems. In Bi-2201 an increase in the hole density (in other words, a decrease in x), also results in a decrease in ρ_c as in the case of Y and La systems. Preliminary measurements on a Pr system with different electron density indicated a similar trend. Recent results^{10,11} on double-layer $\text{Bi}_2\text{Sr}_2\text{CaCu}_2\text{O}_8$ ($T_c \approx 85$ K) have shown equally large ρ_c and non-metallic transport, unlike an earlier report¹². We may conclude that ρ_c is non-metallic for most superconducting materials, although the magnitude of ρ_c depends on the crystal structure: Bi_2O_2 and fluorite-type Pr_2O_2 layers block the out-of-plane conduction more effectively than NaCl-type La_2O_2 and Ba_2O_2 layers. Apparently, there is no correlation between T_c and ρ_c .

Band calculations¹³ can explain the very large anisotropy in Bi-related compounds and smaller one in La and Y systems, and the reduction in anisotropy with increase of x in $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$. They predict, however, that the out-of-plane conduction is always metallic, in sharp contrast to the experimental facts, which suggests that the band theory cannot be applied to the normal state of superconducting copper oxides. To our knowledge, no other theoretical models have predicted

the out-of-plane conduction in high- T_c copper oxides except Anderson's resonating-valence-bond (RVB) theory¹⁴. RVB theory proposes an unconventional conduction mechanism between CuO_2 planes, arising from a confinement of charge carriers to the planes. Out-of-plane conduction occurs only when the charge carriers are released—for example, by thermal fluctuations. This leads to a $1/T$ dependence for ρ_c . The non-metallic $\rho_c(T)$ s that we observe do not fit activation- or hopping-type conductivity with exponential T dependences, but exhibit weaker T dependence such as the power law $\rho_c \propto T^{-\alpha}$ with α in the range $0 < \alpha < 2$, depending on carrier density. This behaviour is evidence for unconventional electrical transport between CuO_2 planes and supports the basic idea of RVB theory, although the theory is insufficient to describe the observed dependence on T .

The metallic ρ_c has been observed in two cases so far. Heavily doped nonsuperconducting $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$ exhibits $\rho_c(T) \propto T^2$ over a wide temperature range below 300 K. Considering that the in-plane resistivity of this compound also shows a quadratic T dependence, which is typical of an electron-electron scattering mechanism in the Fermi liquid state, the same conduction mechanism may be working in all directions and an anisotropic three-dimensional metallic state realized in the heavily doped material. On the other hand, ρ_c in fully oxygenated $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ seems to fit the empirical expression $\rho_c(T) = a + bT$ with anomalously large a ($\sim 10^{-3} \Omega \text{ cm}$). Although the linear term in T is indicative of metallic conduction, large a is not compatible with the conventional transport theory. In this sense, ρ_c in $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$ is still anomalous as in the reduced compounds.

Metallic conduction in all three dimensions is realized in anisotropic system with a spheroidal Fermi surface when $\rho_{ab}\rho_c < \rho_M^2$, where $\rho_M \approx h/(e^2 k_F)$ is the reciprocal minimum metallic conductivity, k_F being the equatorial radius. This is an extension of the Mott-Ioffe-Regel criterion for metallic conductivity in an isotropic system¹⁵. In this sense, high- T_c superconductors in which electrical conduction is metallic in the a - b plane, but non-metallic along the c axis, cannot be conventional three-dimensional anisotropic metals. Specifically, in our study the

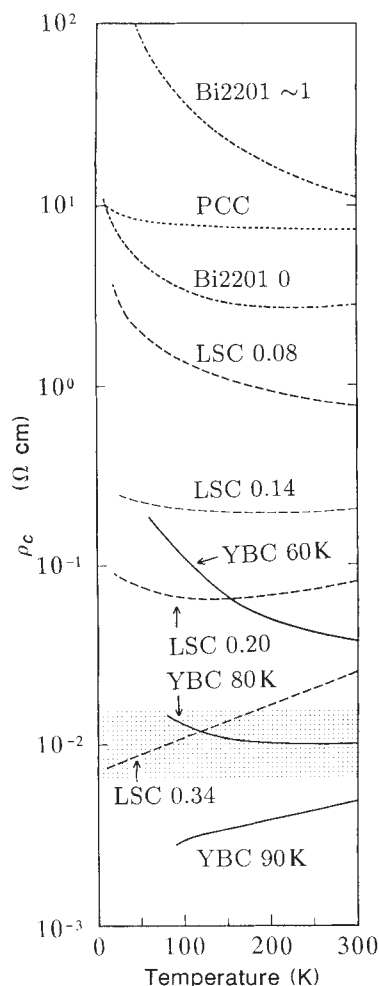


FIG. 2 Temperature dependences of ρ_c for $\text{YBa}_2\text{Cu}_3\text{O}_{7-y}$, $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$, $\text{Pr}_{2-x}\text{Ce}_x\text{CuO}_{4-y}$ ($x \approx 0.15$) and $\text{Bi}_2\text{Sr}_{2-x}\text{La}_x\text{CuO}_6$, abbreviated to YBC, LSC, PCC and Bi2201, respectively. The numbers following LSC and Bi2201 indicate the Sr and La composition x , respectively (the value of $x = 0.14$ for LSC is from ref. 6). The shaded region indicates the critical resistivity along the c axis, which is the extended Mott-Ioffe-Regel limit estimated from the values given in the text.

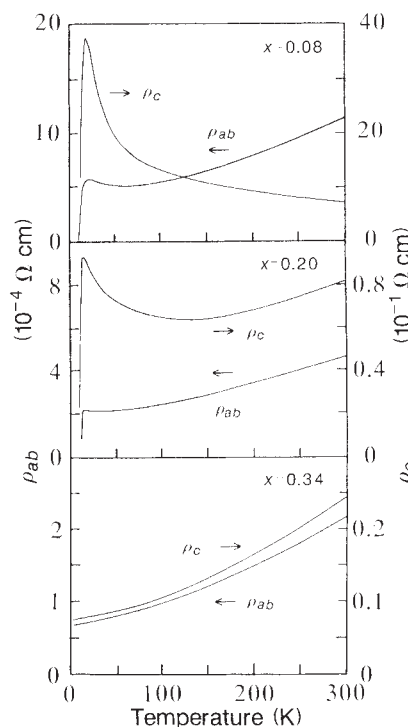


FIG. 3 Temperature dependences of ρ_{ab} and ρ_c for three compositions of $\text{La}_{2-x}\text{Sr}_x\text{CuO}_4$.

materials with negative $d\rho_c/dT$ have $\rho_c \geq 10^{-2} \Omega \text{ cm}$, as seen in Fig. 2. As $\rho_{ab} \approx 10^{-4} \Omega \text{ cm}$ for optimally superconducting materials and ρ_M is roughly estimated to be $10^{-3} \Omega \text{ cm}$, corresponding to a carrier density of $1-3 \times 10^{21} \text{ cm}^{-3}$, the condition for metallic conduction, $\rho_{ab}\rho_c < \rho_M^2$, is almost always violated. Apparently, $\text{YBa}_2\text{Cu}_3\text{O}_{7-x}$ is an exception, with $\rho_c < 10^{-2} \Omega \text{ cm}$ and $d\rho_c/dT > 0$. In this material the CuO chains inserted between CuO_2 planes create a $-\text{Cu}-\text{O}-\text{Cu}-\text{O}-$ sequence along the c axis which might be responsible for the exceptional behaviour. $\square$

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Antiferromagnetism and metallic conductivity in $\text{Nb}_{12}\text{O}_{29}$

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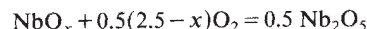
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AT the heart of the controversy about the microscopic origin of superconductivity in high-transition-temperature copper oxide superconductors is the question of whether or not the antiferromagnetism associated with the single-hole $\text{Cu}^{2+} 3d^9$ state is of fundamental importance. To test whether this unconventional spin-mediated superconductivity might be electron/hole symmetric, oxides of the elements with single d -orbital electrons, such as $\text{Ti}^{3+} (3d^1)$, $\text{Nb}^{4+} (4d^1)$ and $\text{W}^{5+} (5d^1)$, are of particular interest. Localized magnetic spin states and magnetic ordering have never been observed previously in transition-metal oxides with one or two electrons in the $4d$ or $5d$ states, because of the preference for conventional d -band metallic conductivity or for metal-metal bonding. In exploring the possibility of a d^1-d^9 and ferroelectricity-superconductivity relationship in oxides (see, for instance, ref. 1), we have found that $\text{Nb}_{12}\text{O}_{29}$, a material with a 'crystallographic shear' structure, displays simultaneously both metallic conductivity, a signature of delocalized electrons, and local-moment magnetism with an antiferromagnetic ordering temperature of 12 K. This suggests that the bonding to oxygen of the $4d$ levels of early-transition-metal elements may not be sufficiently covalent to yield the kind of exotic conductivity (and thus exotic superconductivity) observed for copper oxides.

The superconducting copper oxide perovskites are remarkable for their ability to accommodate a relatively large oxygen deficiency without the breakdown of their crystal structures. For the early transition metal oxides which have three-dimensional blocks of corner-shared MO_6 octahedra as their basic structural component, changing the oxygen stoichiometry causes large-scale structural rearrangement. The blocks are broken along planes of shared corners, and shifted, to eliminate whole planes of oxygen atoms, creating planes of edge-shared octahedra. This 'crystallographic shear' process results in long-range-ordered

arrays of two-dimensional planes of edge-shared octahedra dressing the edges of the three-dimensional corner-shared blocks^{2,3}. The fully oxidized insulating Nb^{5+} oxide, Nb_2O_5 , occurs in many polymorphs with crystallographic shear structures. On chemical reduction of Nb by oxygen removal, a whole series of related crystallographic shear structures is formed. $\text{Nb}_{12}\text{O}_{29}$ is the most stable of the reduced phases occurring between Nb_2O_5 (Nb^{5+}) and NbO_2 (Nb^{4+})^{4,5}. An idealized representation of the crystal structure of the orthorhombic symmetry polymorph of $\text{Nb}_{12}\text{O}_{29}$ is shown in Fig. 1. In the real crystal structure, the niobium-oxygen octahedra are irregular in bond length and bond angle; this is characteristic of ferroelectric-like structural distortions^{6,7,8}. The corner-shared blocks are $3 \times 4 \times \infty$ octahedra in size, and the planes of edge-shared octahedra intersect at right angles along the block boundaries, in a honeycomb array. The structure therefore has both two-dimensional and three-dimensional substructures.

Although small amounts of Nb-based crystallographic shear structures have been synthesized for structural or thermodynamic studies, they have apparently never been synthesized in sufficient quantity or purity to study their physical properties. We have found that they can be synthesized easily and reproducibly by a Nb-gettering technique. Half-gram pellets of Nb_2O_5 (Alfa Inorganics, Puratronic grade) were first sintered at 1,375 °C in air overnight. These were then sealed in evacuated quartz tubes with Nb metal powder (~ 200 mesh) which was prevented from coming in direct contact with the Nb_2O_5 pellets by quartz tube spacers. $\text{Nb}_{12}\text{O}_{29}$ was prepared at 900 °C, 1,200 °C and 1,300 °C by employing Nb: Nb_2O_5 weight ratios between 0.025:1 and 0.035:1. The annealing time was 4 days. At lower Nb: Nb_2O_5 ratios, Nb_2O_5 , $\text{Nb}_{25}\text{O}_{62}$, $\text{Nb}_{47}\text{O}_{116}$ and $\text{Nb}_{22}\text{O}_{54}$ could be made single phase, and at higher ratios, NbO_2 was found to be mixed with the $\text{Nb}_{12}\text{O}_{29}$. The Nb:O ratios of the final products were determined by measuring the change in weight on oxidation of part of the samples at 800 °C in air to Nb_2O_5 :



Single-phase $\text{Nb}_{12}\text{O}_{29}$ was found for samples with O: Nb ratios of 2.41 (± 0.01):1, which agreed within experimental resolution

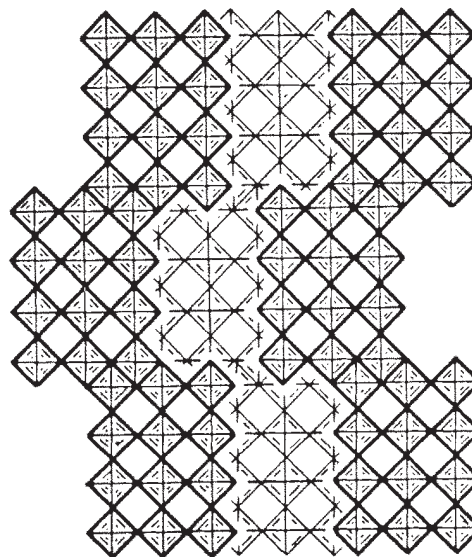


FIG. 1 The idealized crystal structure of orthorhombic $\text{Nb}_{12}\text{O}_{29}$. Octahedra of NbO_6 are shown. Bold octahedra are on a level $1/2$ octahedron height above the background octahedra. The structure repeats infinitely perpendicular to the plane of the figure, to form long columns of corner-shared octahedra dressed by planes of edge-shared octahedra. The planes of edge-shared octahedra occur in the structure where the bold and background octahedra meet, and extend perpendicular to the plane of the paper.

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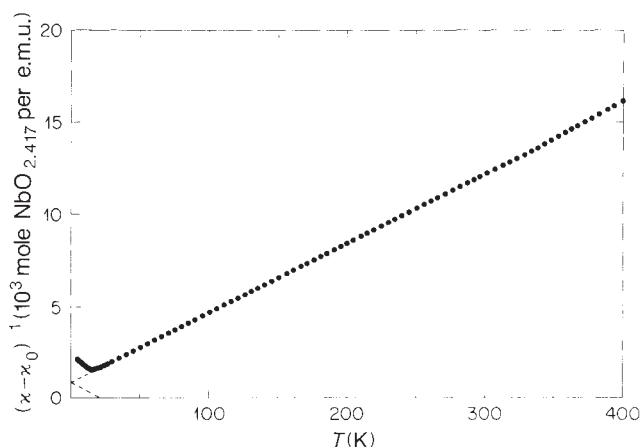


FIG. 2 The inverse magnetic susceptibility of $\text{Nb}_{12}\text{O}_{29}$ measured in a field of 10 kOe, plotted per mole $\text{NbO}_{2.417}$. A small constant term, $\chi_0 = 9.3 \times 10^{-6}$ e.m.u. per mole, has been subtracted. From the linear extrapolation, an interaction temperature θ_{CW} of 24 K is deduced.

with the expected 2.417:1. Powder X-ray diffraction patterns of single-phase material were an excellent match to published patterns^{7,8} of orthorhombic $\text{Nb}_{12}\text{O}_{29}$ and isostructural $\text{Ti}_{12}\text{Nb}_{10}\text{O}_{29}$. Further details of materials preparation and identification will be published elsewhere.

Magnetic susceptibility was measured in a field of 10 kOe in a commercial SQUID magnetometer. In Fig. 2 we present the inverse magnetic susceptibility of $\text{Nb}_{12}\text{O}_{29}$ between 5 and 400 K, plotted per mole $\text{NbO}_{2.417}$ to normalize to 1 Nb per formula unit. For magnetic systems with localized moments, the magnetic susceptibility above the ordering temperature is described in terms of the formula $\chi = \chi_0 + C/(T + \theta_{\text{CW}})$, where C is the Curie constant and θ_{CW} is the Curie-Weiss temperature. θ_{CW} is zero for non-interacting spins, positive for antiferromagnetic spin interactions and negative for ferromagnetic spin interactions. All samples tested showed a small temperature-independent contribution to the susceptibility (χ_0) of $\sim 1.0 \times 10^{-5}$ e.m.u. per mole $\text{NbO}_{2.417}$, which we attribute to the near cancellation of core diamagnetism, Van Vleck contributions, and the paramagnetism of the free carriers. For the sample in Fig. 2, $\chi_0 = 9.3 \times 10^{-6}$ e.m.u. per mole $\text{NbO}_{2.417}$, which has been subtracted from the data before plotting. The data show the Curie-Weiss behaviour expected for antiferromagnetic interactions. Fitting from 30 to 400 K yields $C = 2.67 \times 10^{-2}$ e.m.u. K per mole and

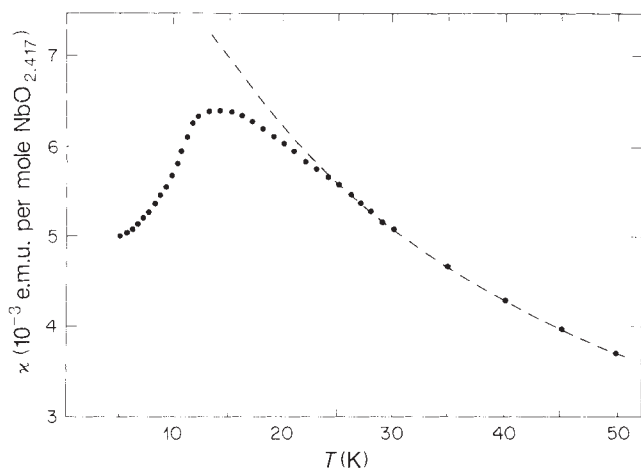


FIG. 3 Detail of the magnetic susceptibility of $\text{Nb}_{12}\text{O}_{29}$ in the region of the antiferromagnetic ordering temperature, plotted per mole $\text{NbO}_{2.417}$. The dashed line shows the extrapolation of the Curie-Weiss fit from high temperatures. Note the deviation from the Curie-Weiss behaviour beginning near 25 K and the ordering transition near 12 K.

a Curie-Weiss temperature of $\theta_{\text{CW}} = 24$ K, which is characteristic of relatively weak interactions. The variation of θ_{CW} from sample to sample is about ± 1.5 K. The antiferromagnetic ordering is shown by the departure of $1/\chi$ from linearity at low temperatures.

Figure 3, in which χ is plotted against T , shows the antiferromagnetic transition in detail. The extrapolation of the Curie-Weiss behaviour at higher temperatures is shown by the dashed line. The data show that the antiferromagnetic transition occurs at 12 K, and also that deviation from the Curie-Weiss behaviour occurs for temperatures up to 25 K. In the simplest models, the antiferromagnetic ordering temperature is expected to be close to θ_{CW} , but it is usually found to be lower. The fact that the antiferromagnetic fluctuations are observed to begin near θ_{CW} but that the actual ordering does not occur until the temperature is reduced to $\sim 0.5\theta_{\text{CW}}$ suggests that either frustration of spins or two-dimensional interactions are involved in determination of the actual ordering temperature.

Resistivity was measured at constant current in four-probe bar geometry. The temperature-dependent resistivity of polycrystalline $\text{Nb}_{12}\text{O}_{29}$ is shown in Fig. 4. The resistivity decreases from $\sim 4 \times 10^{-3}$ to 2×10^{-3} Ω cm as temperature decreases from 300 to 50 K, where it begins to flatten out. Between 30 and 25 K, the temperature range for which χ begins to deviate from Curie-Weiss behaviour, the resistivity begins to drop, finally showing a sharper drop near 12 K (main figure), the antiferromagnetic ordering temperature. Thus spin-induced scattering of the conduction electrons makes a significant contribution to the resistivity above the ordering temperature. In the $3d^1$ antiferromagnetic compounds Sr_2VO_4 (refs 9, 10) and CeTiO_3 (ref. 11) the resistivity is unaffected (semiconducting Sr_2VO_4) or there is a metal-semiconductor transition (CeTiO_3) at the magnetic ordering temperature.

For $\text{Nb}_{12}\text{O}_{29}$, the average Nb valence is 4.83. If the charges were localized, the material would be an insulator and would be written $\text{Nb}_2^{4+}\text{Nb}_{10}^{5+}\text{O}_{29}$. The configuration of Nb^{4+} is $s = 1/2$, $4d^1$. $\text{Nb}_{12}\text{O}_{29}$ is a metallic conductor, however, and remains metallic below the magnetic ordering temperature. The moment calculated from χ as a function of T is only 42% of the value expected if all Nb^{4+} had the full magnetic moment, assuming for simplicity $s = 1/2$, $g = 2$. These results suggest that $\text{Nb}_{12}\text{O}_{29}$ could be an itinerant antiferromagnet. Itinerant antiferromagnets, however, do not show as distinctive a Curie-Weiss behaviour as we have observed for $\text{Nb}_{12}\text{O}_{29}$, which is a signature of localized spins. The complexity of the crystal structure suggests an alternative interpretation: one particular set of Nb

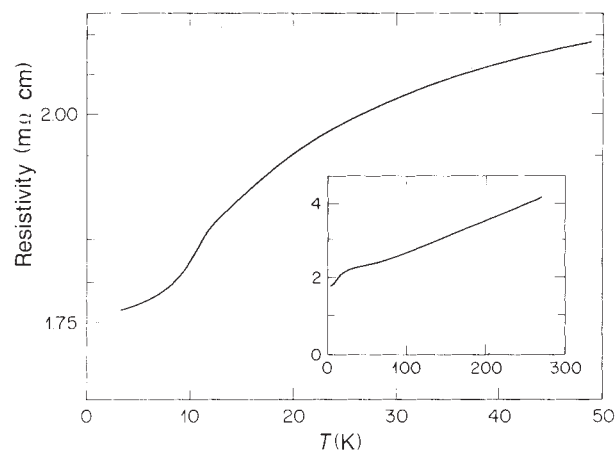


FIG. 4 Detail of the temperature-dependent resistivity of polycrystalline $\text{Nb}_{12}\text{O}_{29}$ in the vicinity of the antiferromagnetic ordering temperature. Inset: the temperature-dependent resistivity over the temperature range 3–300 K.

positions contains Nb of valence 4 with a localized $4d^1$ configuration, giving rise to the magnetism, and other sites contribute delocalized electrons, giving rise to the conductivity. Antiferromagnetism and conductivity in $\text{Nb}_{12}\text{O}_{29}$ could therefore be analogous to ferromagnetism and superconductivity in materials such as ErRh_4B_4 , involving different subsets of electrons.

Our evidence shows that $\text{Nb}_{12}\text{O}_{29}$ is a metallic antiferromagnet with an ordering temperature of 12 K. The resistivity measurements indicate that it is metallic but not superconducting down to 0.25 K. To the best of our knowledge, it is the first niobium-oxide-based magnetic material. The mixed dimensionality of the crystal structure apparently affects the magnetic properties through the reduction of the true ordering temperature to half of θ_{CW} . We have found that the oxides $\text{Nb}_{25}\text{O}_{62}$, $\text{Nb}_{47}\text{O}_{116}$ and $\text{Nb}_{22}\text{O}_{54}$ derived by reduction of Nb_2O_5 also display Curie-Weiss behaviour in plots of χ against T , but do not magnetically

order down to 2 K. Their θ_{CW} s of 0, 7 and 12 K indicate the increasing strength of antiferromagnetic interactions as the Nb^{4+} content increases. Thus, antiferromagnetic interactions are a general property of reduced Nb oxides with crystallographic shear structures. Although some reduced Ti and V oxides based on 3d shells are antiferromagnetic¹², 4d- and 5d-based reduced Mo and W oxides do not display any tendency towards local-moment magnetism. The occurrence of such local moments in reduced Nb 4d-based oxides suggests the existence of narrower electronic bands and poorer metal-oxygen covalency than occur in their molybdenum and tungsten oxide counterparts. Further research is necessary to determine the degree of localization of the magnetic states. More detailed study of the magnetic niobium oxides will be of interest in its own right, and may shed light on the relationships between ferroelectricity, antiferromagnetism and covalency in transition metal oxides. □

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Superconductivity at 18 K in potassium-doped C_{60}

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THE synthesis of macroscopic amounts of C_{60} and C_{70} (fullerenes)¹ has stimulated a variety of studies on their chemical and physical properties^{2,3}. We recently demonstrated that C_{60} and C_{70} become conductive when doped with alkali metals⁴. Here we describe low-temperature studies of potassium-doped C_{60} both as films and bulk samples, and demonstrate that this material becomes superconducting. Superconductivity is demonstrated by microwave, resistivity and Meissner-effect measurements. Both polycrystalline powders and thin-film samples were studied. A thin film showed a resistance transition with an onset temperature of 16 K and essentially zero resistance near 5 K. Bulk samples showed a well-defined Meissner effect and magnetic-field-dependent microwave absorption beginning at 18 K. The onset of superconductivity at 18 K is the highest yet observed for a molecular superconductor.

The sensitivity to air of alkali-metal-doped fullerenes (A_xC_n) limits the choice of sample preparation and characterization techniques. To avoid sample degradation, we carried out reactions with the alkali metal vapour and C_{60} in sealed tubes either in high vacuum or under a partial pressure of helium. The C_{60} was purified by chromatography¹ of fullerite² and was heated at 160 °C under vacuum to remove solvents.

Small amounts of the individual fullerenes (~0.5 mg) were placed in quartz tubes with alkali metals and sealed under vacuum. These samples were subjected to a series of heat treatments and tests for superconductivity by 9-GHz microwave-loss experiments⁵. Preliminary tests indicated that only the K-doped C_{60} showed a response consistent with a superconducting transition (Fig. 1). For this reason, together with the fact that K_xC_{60} showed the highest film conductivity¹, we focused our studies on the K-doped compound.

The conductivity measurements were performed on potassium-doped films of C_{60} that were prepared in a one-piece all-glass version of the apparatus described previously⁴. This reaction vessel was sealed under a partial pressure of helium

before reaction. This configuration allowed both *in situ* doping and low-temperature studies of thin films. All measurements were made in a four-terminal Van der Pauw configuration using a 3- μA a.c. current at 17 Hz. Figure 2 shows the temperature dependence of the resistivity of a 960-Å-thick K_xC_{60} film. The film was doped with potassium until the resistivity had fallen to $5 \times 10^{-3} \Omega \text{ cm}$. The resistivity increases by a factor of two on cooling the sample to near 20 K. Below 16 K, the resistivity starts to decrease; zero resistivity ($<10^{-4}$ of the normal state) is obtained below 5 K. The 10-90% width of the transition is 4.6 K. At 4 K we measured the lower bound to the critical current to be 40 A cm^{-2} .

A bulk polycrystalline sample of nominal composition K_3C_{60} was prepared by reaction of 29.5 mg of C_{60} with 4.8 mg potassium. The amount of potassium was controlled volumetrically by using potassium-filled pyrex capillary tubing cut to size in a dry box. The reaction was run with the C_{60} in a 5-mm fused silica tube joined to a larger tube in which the potassium-containing capillary was placed. The tube was sealed after being evacuated and refilled with 10^{-2} torr of helium to serve later as a thermal-exchange gas for low-temperature measurements. With the C_{60} -containing end of the tube at room temperature,

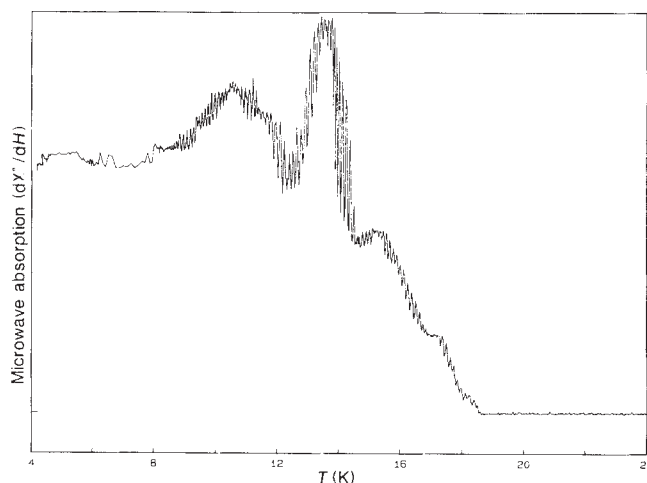


FIG. 1 Microwave loss as a function of temperature for K_xC_{60} in a static field of 20 Oe.

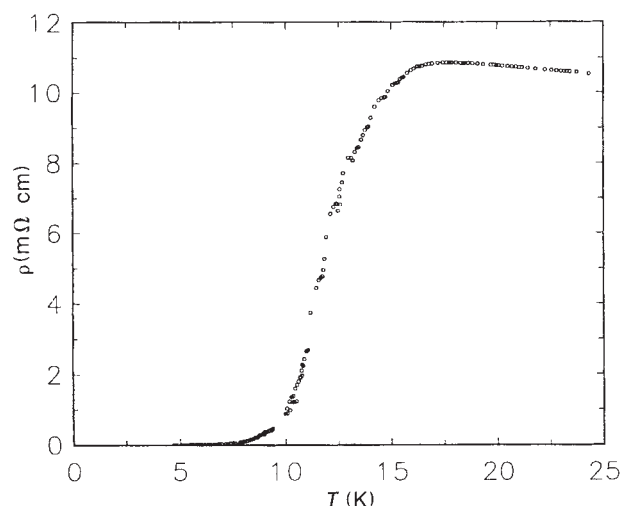


FIG. 2 Temperature dependence of the electrical resistivity of a 960-Å-thick film of K_xC_{60} .

the potassium was distilled from the capillary in a furnace at 200 °C. Some reaction of the potassium with the quartz tube, visible as a dark brown discoloration, was observed at this temperature. Unreacted potassium was observed after this period. Following distillation of the potassium to the C_{60} end, the tube was shortened by sealing to about 8 cm and heated to 200 °C for 36 h. Finally, the tube was resealed to a length of about 4 cm for magnetic measurements.

The temperature dependence of the d.c. magnetization of the sample with nominal composition K_3C_{60} was measured in a SQUID magnetometer (Fig. 3). On zero-field cooling the sample to 2 K, a magnetic field of 50 Oe was applied. On warming, this field is excluded by the sample to 18 K; this verifies the presence of a superconducting phase. The bulk nature of superconductivity in the sample is demonstrated unambiguously by cooling in a field of 50 Oe. A well defined Meissner effect (flux expulsion) develops below 18 K. The shape of the magnetization curve, in particular the temperature-independent signal at low temperature, indicates good superconducting properties for this sample. Also noteworthy is the relatively narrow transition width. The magnitude of the flux exclusion for the zero-field-cooled curve corresponds to 1% volume fraction. This small fraction is possibly due to non-optimal doping or the granular nature of the sample. The large value of the Meissner effect for the field-cooled curve relative to the total exclusion, however,

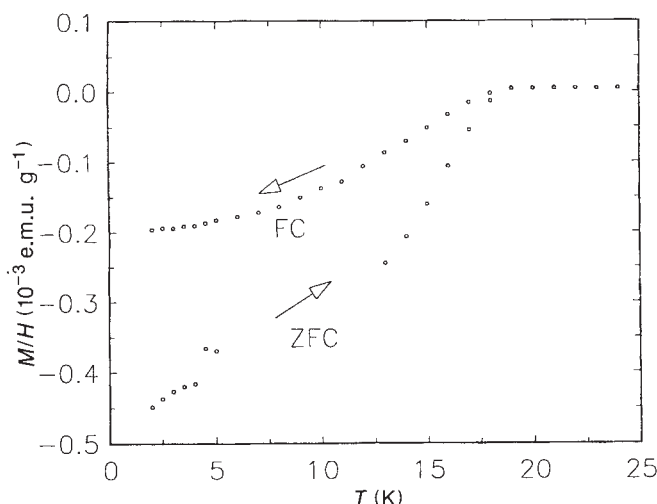


FIG. 3 Temperature dependence of the magnetization of a K_xC_{60} crystalline sample. The direction of temperature sweep in the field-cooled (FC) and the zero-field-cooled (ZFC) curves is indicated by the arrows.

indicates bulk superconductivity in the electrically connected regions.

The universally accepted tests for superconductivity, namely a transition to zero resistance and a Meissner effect showing the expulsion of magnetic field, demonstrate unequivocally the existence of superconductivity in K_xC_{60} . The 18-K transition temperature is the highest yet reported for a molecular superconductor. This may be compared with the previously reported occurrence of superconductivity at 0.55 K in potassium-intercalated graphite⁶. We expect that optimization of composition and crystallinity will lead to further improvement in the superconducting properties. □

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Sedimentation rates, residence times and radionuclide inventories in Lake Baikal from ^{137}Cs and ^{210}Pb in sediment cores

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RADIONUCLIDES in lake sediments may act as indicators of the sedimentation rate of particles on which they are adsorbed; these rates in turn provide a direct indication of the residence times of particles in the water column. The radionuclide ^{137}Cs is anthropogenic (an atomic-bomb product), so that its concentration in sediments also reveals the input history of this species and thus a record of atmospheric contamination by this nuclide in the lake's watershed. Here we report measurements of ^{137}Cs and the natural radionuclide ^{210}Pb in cores from several stations throughout the three basins of Lake Baikal. The results confirm earlier indirect estimates¹ of the mean sedimentation rate, and show that the effective settling rate of these radionuclides is the same as that in the Great Lakes; the longer residence times for Lake Baikal are therefore simply a consequence of its greater depth. As well as allowing estimates of fluxes at the sediment-water interface²⁻⁴, our results provide information on the timing of palaeolimnological events⁵, on the existence of different depositional zones throughout the lake, on the long-term (decadal) diffusion of nuclides in sediments⁶ and for the development of mass-balance models for sediments and contaminants⁷⁻⁹.

Caesium-137 and lead-210 (half lives of 30.2 and 22.3 years, respectively) have been measured in 10 sediment cores collected from throughout Lake Baikal (Fig. 1). Each ^{210}Pb profile (Fig. 2) shows a layer of constant activity (surficial mixing) followed by an exponential decrease in concentration with depth to a constant value maintained by *in situ* decay of ^{226}Ra (ref. 2).

TABLE 1 Sedimentation rates, mixing depths and inventories of ^{210}Pb and ^{137}Cs in Lake Baikal sediments

Station	Location		Depth (m)		Sedimentation rates*		Mixing depth (yr)	Inventories (Bq cm^{-2})		
	Lat. ° 'N	Long. ° 'E			Linear (cm yr^{-1})	Mass ($\text{g cm}^{-2} \text{yr}^{-1}$)		^{210}Pb	^{137}Cs	$^{137}\text{Cs}/^{210}\text{Pb}$
1	51 47	104 28	1340	(a)	0.074	0.022	10	0.27	0.14	0.52
				(b)	0.061	0.018				
2	51 44	105 14	1510	(a)	0.072	0.016	40	0.34	0.13	0.38
				(b)	0.043	0.008				
3	52 23	106 09	280	(a)	0.100	0.028	30	0.51	0.57	1.12
				(b)	0.084	0.022				
4	52 22	106 13	100	(a)	0.121	0.052	20	0.47	0.70	1.50
				(b)	0.091	0.034				
5	52 25	105 59	600	(a)	0.084	0.020	20	0.43	0.26	0.60
				(b)	0.066	0.014				
6	53 11	107 46	1710	(a)	0.042	0.009	70	0.31	0.14	0.45
				(b)	0.032	0.007				
7	53 32	107 58	230	(a)	0.016	0.003	20	0.11	0.040	0.36
				(b)	0.016	0.003				
8	53 55	108 25	930	(a)	0.026	0.006	30	0.14	0.00†	—
				(b)	0.026	0.004				
9	52 55	107 53	900	(a)	0.025	0.008	10	0.13	0.091†	0.70
				(b)	0.024	0.006				
10	52 59	107 51	1300	(a)	0.035	0.008	10	0.096	0.00†	—
				(b)	0.035	0.006				

* For each station there are two values for the sediment accumulation rate: (a) without and (b) with mixing, see text.

† The differences in the ^{210}Pb inventories at these two stations which are very close together on a steep slope (Fig. 1), and the absence of ^{137}Cs at station 10, may be explained by loss of surficial sediment because of slumping.

‡ ^{137}Cs was also not detected in a core collected at this same station in 1990. From the bathymetry, slumping is unlikely at this station.

Sedimentation rates and mixed depths were estimated using the rapid steady-state mixing model (RSSM)^{2,3}. Inclusion of mixing provided the best fit but had little effect on calculated sedimentation rates (Table 1). These varied from a low of 0.016 cm yr^{-1} ($0.003 \text{ g cm}^{-2} \text{yr}^{-1}$) in the northern basin to highs of 0.074 cm yr^{-1} ($0.018 \text{ g cm}^{-2} \text{yr}^{-1}$) in the southern basin and 0.12 cm yr^{-1} ($0.034 \text{ g cm}^{-2} \text{yr}^{-1}$) in the Selenga delta. The mean sedimentation rate from all cores was $0.06 \pm 0.03 \text{ cm yr}^{-1}$ without or $0.05 \pm 0.03 \text{ cm yr}^{-1}$ with mixing. When we excluded stations in the Selenga delta³⁻⁵, mean sedimentation rates were $0.04 \pm 0.02 \text{ cm yr}^{-1}$ and $0.03 \pm 0.01 \text{ cm yr}^{-1}$. When all cores were used, the basin-averaged mean values for the lake were 0.043 and 0.035 cm yr^{-1} .

Mixing depths varied from ~ 0.5 to 2 cm, resulting in an effective time resolution for discrete inputs of ~ 20 to 100 years (ref. 4). At stations 2, 3 and 6, the mixed depth coincided with a thin, hard iron-manganese oxide layer. There was no evidence of increased ^{210}Pb concentrations in this layer. For the RSSM model, mixing is assumed to be rapid enough to homogenize

activity, but at stations 3 and 6 excess ^{210}Pb decreases exponentially in the mixed layer. This is not uncommon in sediments and can be modelled using a finite diffusive mixing rate $\sim 1 \text{ cm}^2 \text{yr}^{-1}$ for station 6 (refs 3, 4, 10 and 11).

With low sedimentation rates, and excess ^{210}Pb confined to a layer $\leq 10 \text{ cm}$ thick, exponential distributions could result from diffusive processes in the absence of net sedimentation, such as mixing by near-bottom turbulence or bioturbation^{4,5}. There is no *a priori* means to establish a case for the dominance of advection, but the radiometric rates are confirmed by independent estimates¹¹. Hutchinson¹² estimated the mean sedimentation rate to be $0.01\text{--}0.02 \text{ cm yr}^{-1}$ based on the age of the lake and the thickness of the sedimentary deposits. Afanasayev¹³ calculated a rate of 0.022 cm yr^{-1} from the inputs of suspended matter to the lake. Goldyorev *et al.*¹⁴ report the rate to be between $0.04\text{--}0.08 \text{ cm yr}^{-1}$ from several indirect methods.

The ^{137}Cs profiles do not exhibit the sub-surface maxima generally associated with the maximum input of fallout in 1963 (Fig. 2)^{2,15}. The activity decreased more or less exponentially

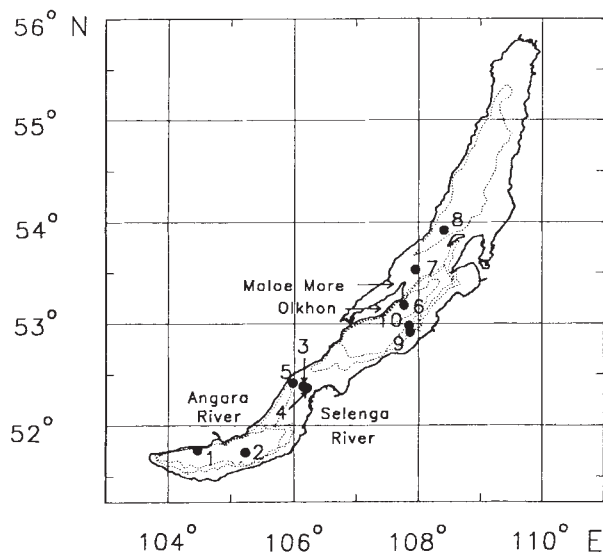
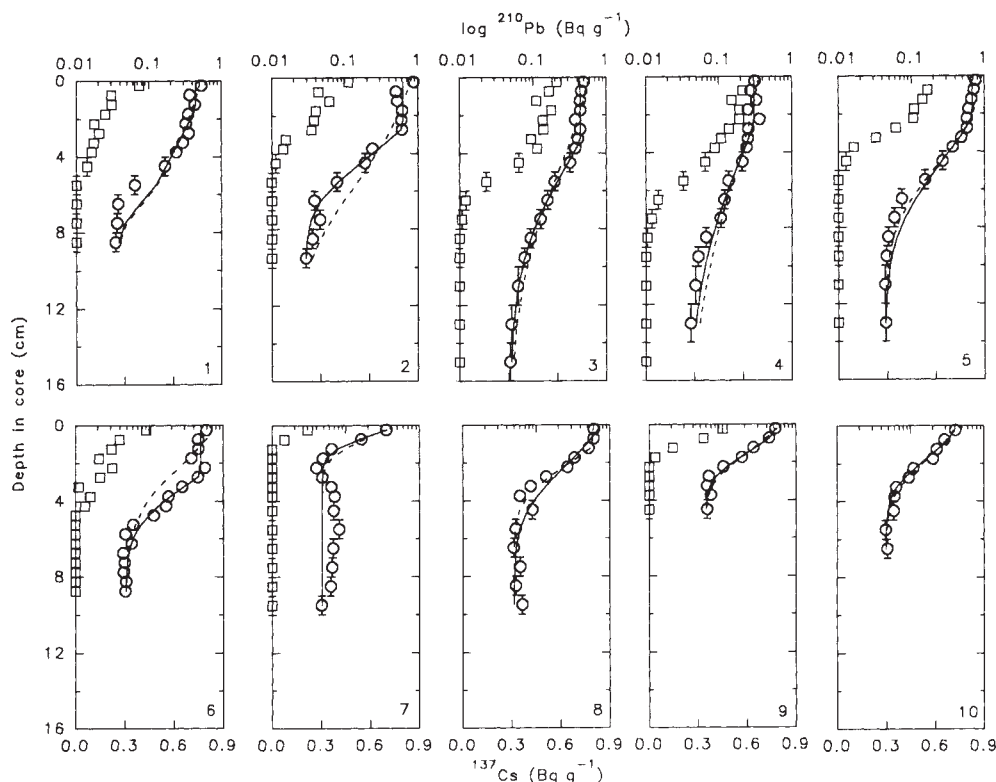


FIG. 1 Lake Baikal and the stations occupied during the 1988 cruise. Isobaths are drawn at 800, 1,200 and 1,600 m. There are three basins, north, central and south with maximum depths of 900, 1,700 and 1,500 m respectively¹. A delta formed by sediments from the Selenga River, with a maximum depth of 500 m, marks the boundary between the central and south basins. Over 60% of the input of water enters the lake through the Selenga River. Water flows out of the lake only through the Angara River with a hydraulic replacement time of 400 years. Gravity cores (7.5-cm diameter, $\sim 80\text{-cm}$ long) were collected at stations representative of the sedimentary regimes in the three basins and the Selenga delta encompassing water depths from 100 to 1,700 m. An undisturbed oxic sediment-water interface was obtained for all cores sectioned on board for later analysis. The sediments generally contain $\leq 3\%$ of organic carbon.

FIG. 2 Observed depth profiles for ^{210}Pb (circles) and ^{137}Cs (squares) in Bq g^{-1} dry weight obtained from non-destructive gamma-ray analysis³³ and isotope dilution alpha spectrometry². The symbols and vertical bars represent sampling intervals of 0.5 and 1.0 cm respectively. The solid and dashed lines represent the best fits to these data using mass sedimentation rates estimated by the RSSM model with and without mixing respectively. The ^{210}Pb concentration decreases to an essentially constant value in ~ 110 years (five half-lives).



with depth from the sediment-water interface, even when a sub-surface peak would have been expected. If ^{210}Pb -derived sedimentation rates and mixing depths are correct, then the observed profiles cannot be attributed to redistribution of sediment by mixing, but may result from a longer residence time of ^{137}Cs in the water than in other lakes, and/or from post-depositional mobility by molecular-scale diffusion. Both processes depend on the affinity of ^{137}Cs for particles as measured by a distribution coefficient, K_d (concentration on particles divided by concentration in water, expressed in ml g^{-1}). The value of K_d depends on suspended particle concentration¹⁶ and lake mineralogy, with $K_d \geq 10^5$ for lakes containing clay minerals that irreversibly bind ^{137}Cs (refs 17, 18, 19), and $K_d \leq 1,000$ for organic-rich, mineral-deficient lakes^{20,21}. Laboratory measurements using Baikal sediments at concentrations of 0.0022 and 220 g l^{-1} (porosity 0.91), corresponding to overlying and sediment pore water give $K_d > 10^5$ and $K_d > 10^4$ respectively, similar to values measured for the Great Lakes¹⁸.

Calculations indicate that although diffusional migration of ^{137}Cs may produce some smoothing and penetration, the absence

of a sub-surface maximum is largely the result of a long aquatic residence time, T_r . If Φ and Φ^* ($\text{Bq cm}^{-2} \text{yr}^{-1}$) are the fluxes of ^{137}Cs to the lake surface and sediments, respectively, then

$$d\Phi^*/dt = [(1/T_r) \times \Phi] - [\lambda_{\text{Cs}} + (1/T_r)] \times \Phi^* \quad (1)$$

where the radioactive decay constant $\lambda_{\text{Cs}} = \ln 2/30.2 \text{ yr}^{-1}$. Input fluxes, Φ , for Baikal are unknown, but are assumed to be proportional to the well-known loadings to the Great Lakes^{22,23}. We calculated the fluxes to the sediments, Φ^* , for a series of values of T_r (Fig. 3a). These fluxes are the input to the RSSM model or, more correctly, an advection-diffusion model that includes post-depositional mobility by means of reversible exchange between solid and liquid phases²⁴, and is extended to calculate the relative distribution of ^{137}Cs in sediments with and without mixing²⁵. The characteristic penetration depth of ^{137}Cs into sediments by molecular-scale diffusion is

$$z^* = (4 \times D_e \times t^*)^{1/2} \quad (2)$$

where D_e ($0.056 \text{ cm}^2 \text{yr}^{-1}$) is the effective ^{137}Cs diffusion coefficient in pore water, and t^* is the time since the main input

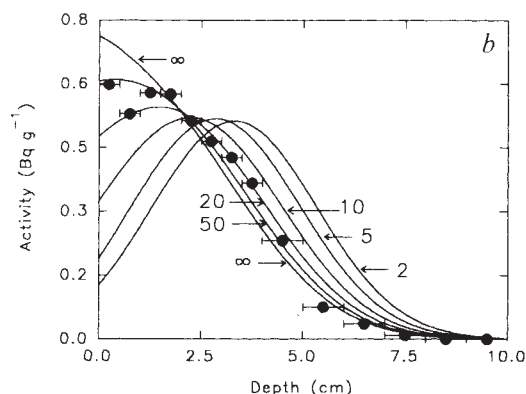
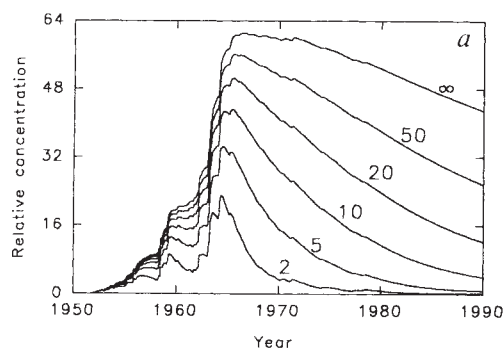


FIG. 3 Variation in the concentration of ^{137}Cs as a function of particle residence time in water ($T_r = 2, 5, 10, 20, 50$ and ∞), a, in water (Φ^*) as a

function of time, b, in sediments as a function of depth (see text). The solid circles represent the observed profile for ^{137}Cs at station 4.

in the 1960s (~ 25 yr). D_e is a function of porosity (0.8) and of K_d (10^4), refs 24 and 25. As $z^* \leq 2$ cm, molecular-scale diffusion may account for some, but not all, of the penetration of ^{137}Cs into the sediments.

The ^{137}Cs profile at station 4 can be compared with profiles calculated using the ^{210}Pb sedimentation rate and values of Φ^* for a series of residence times (Fig. 3a) in the advection-diffusion model with no mixing²⁵ (Fig. 3b). The best fit for the residence time is ≥ 20 yr. This is consistent with an independent value calculated from a box model of Baikal, and with the measured $K_d \approx 10^5$ for ^{137}Cs . For a suspended particle concentration $m_p = 1 \text{ mg l}^{-1}$ (ref. 14) the fraction on particle is²⁶

$$f_p = (m_p \times K_d) / (1 + m_p \times K_d) \approx 0.1 \quad (3)$$

Then for a mean depth $h = 740$ m (ref. 1) and a mean particle settling rate $v_p \approx 1 \text{ m d}^{-1}$ (ref. 14),

$$T_r = h / (f_p \times v_p) \leq 20 \text{ yr.} \quad (4)$$

This is an upper limit; K_d will be higher in natural systems because equilibration times are longer.

The shape of the ^{137}Cs profiles in Lake Baikal therefore results largely from longer aquatic residence times. For the Great Lakes, the K_d for ^{137}Cs , $\sim 5 \times 10^5$ (ref. 18), is similar to that in Baikal, and values of m_p and v_p are almost identical²⁷. Residence times for plutonium in the five interconnected Great Lakes are directly proportional to the mean lake depth²⁷. Plutonium has a similar history of inputs, and K_d (P_u) $\approx K_d$ (^{137}Cs). The linear relationship extrapolates well to Lake Baikal, yielding a mean apparent settling rate ($f_p v_p$) of 0.1 m day^{-1} for all of the lakes. Thus the difference in ^{137}Cs residence times between Baikal and the Great Lakes reflects the extraordinary depth of Lake Baikal and not the affinity of ^{137}Cs for particles.

At steady state, the inventory of excess ^{210}Pb in the water is the difference between the atmospherically supported inventory, I_{atm} , and the basin-weighted sedimentary inventory, I_{sed} (0.19 Bq cm^{-2} , Table 1). The removal rate is equal to the steady-state flux to the sediments,

$$\Phi_{\text{sed}} = I_{\text{sed}} \times \lambda_{\text{Pb}} \quad (5)$$

($\lambda_{\text{Pb}} = \ln 2 / 22.3 \text{ yr}^{-1}$), then from equation (1) with $d\Phi/dt = 0$ the residence time in the water is given by

$$T_r = (I_{\text{atm}} - I_{\text{sed}}) / (I_{\text{sed}} \times \lambda_{\text{Pb}}) \quad (6)$$

The atmospheric flux of ^{210}Pb to Baikal is unknown, but may be estimated from the concentrations in air²⁸ ($C_{\text{atm}} = 0.47$ and $0.32 \text{ Bq per } 1,000 \text{ m}^3$) and rainfall^{23,1} ($x = 7.2$ and 3.4 cm per month) over the Great Lakes (40 – 45°N) and Baikal (50 – 55°N) if we use an empirical relationship²⁹ to calculate relative fluxes

$$\Phi_{\text{dep}} \propto C_{\text{atm}} \times (1 - e^{-0.02x}) \quad (7)$$

and compare their ratio with the measured flux³⁰ over the Great Lakes ($0.027 \text{ Bq cm}^{-2} \text{ yr}^{-1}$). For Baikal, the inventory supported by this flux, I_{atm} , is $\sim 0.30 \text{ Bq cm}^{-2}$ and from equation (5) $T_r \approx 19$ yr. This value is consistent with those already calculated in two different ways from the ^{137}Cs data. As K_d for ^{210}Pb is $> 10^5$ (ref. 26), this result is not unexpected but gratifying considering the assumptions made.

Differences in inventories of excess ^{210}Pb and ^{137}Cs in the basins of Lake Baikal are intriguing and raise questions as to their sources, transport and fate, and by implication, the fate of contaminants with similar chemical properties. Inventories of excess ^{210}Pb vary between 0.096 and 0.51 Bq cm^{-2} with higher values occurring in the deepest areas of the southern and central basins (water depths $\geq 1,500$ m) and in the Selenga delta. Outside the northern basin, the relative inventories of ^{137}Cs correlate

well with those for ^{210}Pb (0.13 – 0.70 Bq cm^{-2}). Several cores taken in 1990 confirm that there is little or no ^{137}Cs in sediments throughout the northern basin. This result suggests that either there was little input from the atmosphere or rivers into northern Baikal or that, in keeping with the lower ^{210}Pb sedimentation rates at stations 7 and 8, there is a strong north-to-south transport of particles.

Elsewhere in the lake there are strong correlations between sedimentation rates and inventories, and between inventories, of both radionuclides. Away from the Selenga River delta the ratio of inventories of ^{210}Pb and ^{137}Cs is usually ≤ 0.7 , whereas close to the delta the ratio is > 1.0 . The inventories of ^{210}Pb are consistent with the estimated inputs, but ^{137}Cs inventories at most stations are less than predicted for this latitude from worldwide monitoring^{28,31} ($\sim 0.20 \text{ Bq cm}^{-2}$). The low inventories for ^{137}Cs in the lake as a whole and the high ^{137}Cs : ^{210}Pb ratios near the Selenga River suggest that either sediments from the river preferentially scavenge ^{137}Cs , or this river is an important source of ^{137}Cs to the delta area. The latter would be unusual because generally only a small fraction of the total inventory in a watershed is mobilized into rivers and transported downstream, with the movement decreasing with time³². But the Selenga River watershed extends into an area of Mongolia directly downwind of Chinese nuclear testing sites used in the 1970s, and continuing inputs of ^{137}Cs from the river cannot be ruled out. $\square$

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Late Palaeozoic deformation in central Africa: a result of distant collision?

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SEISMIC reflection profiles from the Cuvette Centrale (Central Basin) of Zaire show that a major contractional deformation affected a part of central Africa during the late Permian and early Triassic periods. At the same time, other regions of central Africa experienced extensional and strike-slip deformation and associated rift formation. The coexistence of such varied styles of tectonic activity over a large continental area resembles the Cenozoic tectonics of central Asia and northwest Europe. The intracontinental deformation seen in these latter areas is attributed to collisional processes at a distant continental margin¹⁻³. By analogy, we argue here that deformation of central Africa in the late Palaeozoic period was a direct result of collisional processes some distance away at the southern margin of Gondwana.

The late Palaeozoic evolution of central Africa has been characterized as a period of Permo-Triassic rifting followed by a widespread magmatic event during the early Jurassic period^{4,5}. These events are commonly believed to mark the early stages of the breakup of Gondwana, and rifts of similar age have been described in Madagascar, India, Australia and South America. The rift basins are characterized by a combination of extensional and strike-slip tectonics⁶ and a stratigraphy comparable to the Karoo Supergroup of South Africa⁷.

Some 2,900 km of reprocessed seismic reflection data from the Cuvette Centrale of Zaire (Fig. 1) reveal a new dimension to this late Palaeozoic evolution. Figure 2 shows a portion of a regional seismic reflection profile across the Cuvette together with a geological interpretation of the whole profile. The section comprises a central basement high (the Kiri High) flanked by deep sedimentary basins. The sparse seismic grid shows that the high follows a trend from WNW to ESE across the basin and is bounded to the northeast and southwest by thrusts which give the uplift a bipolar geometry. Five stratigraphic sequences are identified from the seismic, well and outcrop data in the Cuvette (Fig. 3): a late Proterozoic carbonate-evaporite sequence (I), a Cambrian marine clastic sequence (II), an Ordovician to Devonian marine clastic sequence (III), a Permo-Carboniferous glaciogenic sequence (IV) and a Triassic-Recent continental sequence (V). Marked truncational unconformities occur between the Cambrian and Ordovician sequences and between the Permo-Carboniferous and Triassic-Recent sequences. The seismic data show both of these unconformities to be a result of compressional tectonics focused on either side of the Kiri High (Fig. 2). The older of the events correlates temporally and spatially with the Pan-African Tectonothermal event⁸. The younger is a previously unrecognized phase of 'late Palaeozoic' compressional tectonics.

The seismic data show the major elevation and marked truncation below the uppermost sequence resulting from the late Palaeozoic event (Fig. 2). The geological section interprets the elevation and associated monoclinical structure as a passive roof to SSW-directed basement thrusts. The age of this deformation is derived from a combination of well and outcrop data⁹. The Dekese well (Fig. 1), in the south of the basin, intersected a late Carboniferous/early Permian glacial sequence, known regionally as the Lukuga Formation, which is deformed below the regional late Palaeozoic unconformity¹⁰. The unconformity

is immediately overlain by early Cretaceous continental sediments. In the Samba well (Fig. 1) the same late Palaeozoic unconformity is overlain by sediments lithologically correlated with the Stanleyville Formation at outcrop¹¹. The Stanleyville Formation is dated as Oxfordian to Aptian¹². These well data constrain the deformation as later than early Permian but before late Jurassic.

Further age constraints on the unconformity exist in the outcrop geology of the eastern margin of the basin. A marked angular unconformity, correlated with the seismically defined late Palaeozoic unconformity, is mapped between the Lukuga Formation and the overlying Haute Lueki Formation. The Haute Lueki Formation ranges in age from early Triassic to middle Liassic, and passes upwards, with a middle Jurassic hiatus, into the structurally conformable late Jurassic Stanleyville Formation¹³. These seismic and outcrop data date the late Palaeozoic unconformity and associated deformation as between the late Permian and earliest Triassic periods (Fig. 3).

Elsewhere in central Africa the late Permian/early Triassic is marked by the formation of rift basins and associated fluvio-deltaic sedimentation^{14,15}. Coarse clastic sediments were fed axially along the rifts and marked the development of the Karoo rift basins as seen today¹⁵. Before the Triassic, the upper Permian was characterized by widespread lacustrine sediments, with little evidence of a confined rift basin geometry¹⁶. The rifting was followed by a large Liassic volcanic event¹⁷ concentrated in southern and south central Africa. In the middle Jurassic, renewed rifting in East Africa led to the separation of East and West Gondwana.

The late Permian/early Triassic evolution of central Africa is thus characterized by rifting, folding and thrusting events that are coeval, or at least very close in time. The rifts are a result of both extensional and strike-slip displacements that produce a roughly east-west extension. The location and geometry of the rifts are controlled by pre-existing basement structures which were reactivated to facilitate the extension⁶. The compressional tectonics of Zaire produce shortening in the approximate

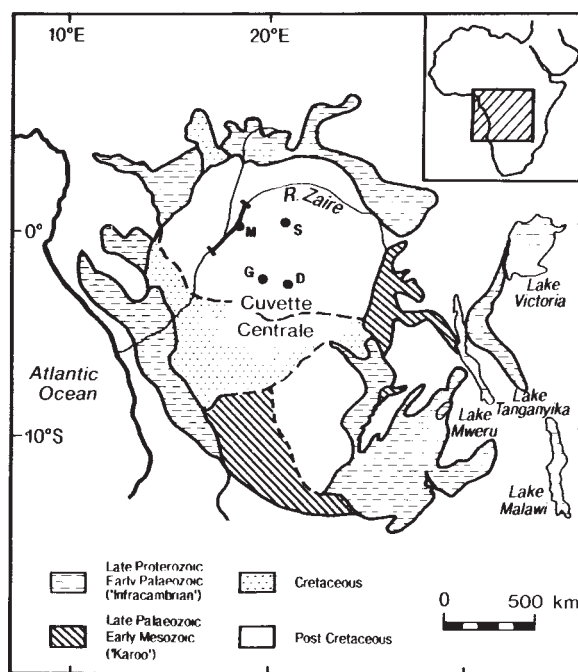


FIG. 1 Sketch map showing the regional geology and location of the Cuvette Centrale of Zaire. The bold line indicates the location of the cross section in Fig. 2. The dots refer to well locations: M, Mbandaka; D, Dekese; S, Samba; G, Gilson.

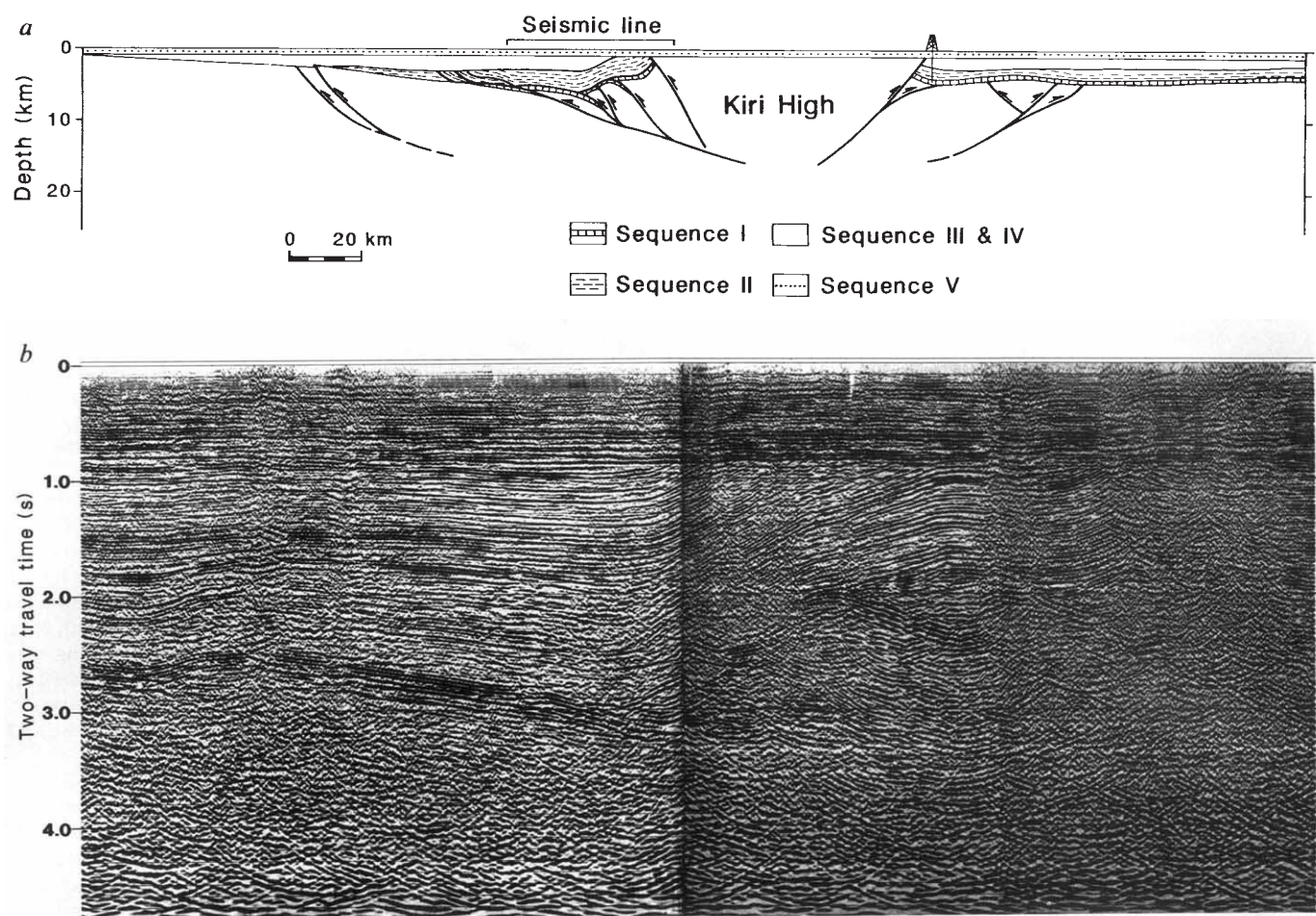


FIG. 2 a, Schematic cross-section through the Cuvette Centrale (vertical scale is twice horizontal scale). b, Seismic data showing the detail of the western margin of the Kiri High. The sequences marked are shown in Fig. 3 and discussed in the text.

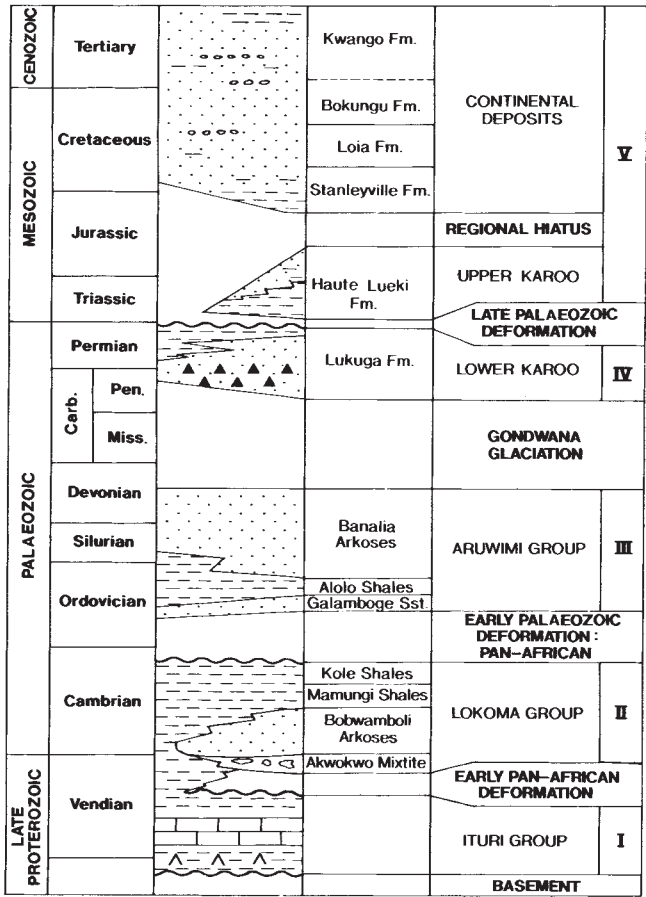


FIG. 3 Schematic chronostratigraphic column outlining the evolution of the Cuvette Centrale. The Roman numerals refer to the sequences discussed in the text.

direction NNE-SSW and also reactivate earlier structures. The compression probably involves a component of strike-slip displacement, but we have no evidence to constrain this. Overall, the extension and compression combine to produce roughly north-south shortening and east-west extension of central Africa. This kinematic compatibility supports the theory of coeval development, which would allow there to be a common cause for the different tectonic environments.

Figure 4 outlines the distribution and nature of the structures thought to be active during this Permo-Triassic deformation. We cannot define the geometry and continuity of the structural pattern and specific fault linkages completely because the stratigraphic record is sparse and cover sediments are widespread. But it is probable that the structural pattern is mainly controlled by pre-existing basement structures inherited from earlier tectonic episodes⁶, and it is therefore unlikely that the pattern is simple or theoretically pleasing.

The active tectonics of central Asia include a similar variety

of tectonic environments^{1,18} generated by continental collisions on the southern margin of Asia, notably the collision with India¹. The effects of this collision are seen more than 3,000 km north of the Indian suture¹⁹. Northwest Europe has been similarly affected by Alpine deformation, more than 1,000 kilometres from the main orogenic front³. By analogy, we argue here that the late Palaeozoic deformation of central Africa is a result of deformational processes acting along the southern margin of Gondwana (Fig. 4). The more obvious effects of this deformation are the Permo-Triassic Cape fold belt and Karoo foreland basin of South Africa, and the Ventana fold belt of South America. Roughly 2,500 km away, the Kiri uplift and associated structures of Zaire seem to be an intracontinental response to this deformation. Nearer the Cape fold belt, but still some 100 km into its foreland, structures similar to those outlined from Zaire have been described in the Karas Mountains of Namibia²⁰ (Fig. 4). In Namibia, Dwyka tillites and glaciomarine sediments of Permo-Carboniferous age are involved in contractional faulting and folding along a northerly trend. The upper age limit on these structures is poorly constrained, but we suggest that they also developed as an intracontinental response to the deformation that produced the Permo-Triassic Cape fold belt.

This example of collision-related intracontinental tectonics in Gondwana involves a plate which had a pre-collisional passive continental margin. Of our two proposed analogues, the situation in northwest Europe is similar to this, whereas central Asia had a pre-collisional active margin. It seems clear that in both cases widespread and variable intracontinental tectonic activity is associated with deformation of the margin. The driving force for the Gondwanan orogenic event is uncertain, although it has been suggested that the Permo-Triassic period was the time of the collision between the Patagonia terrane and Gondwana²¹. Widespread, intracontinental deformation is compatible with this model.

This analysis presents the Cuvette Centrale data in its regional setting and outlines a unifying theme for the late Palaeozoic tectonics of southern and central Africa. It also raises two important questions for the tectonics of Gondwana and for geodynamics in general. First, to what extent do late Palaeozoic compressional deformations of Gondwana remain unrecognized because of a lack of late Palaeozoic and early Mesozoic stratigraphic data? Second, were the collisional processes described above an instigating factor in the breakup of the continent of Gondwana, an event which followed the Permo-Triassic tectonics and early Jurassic volcanism? □

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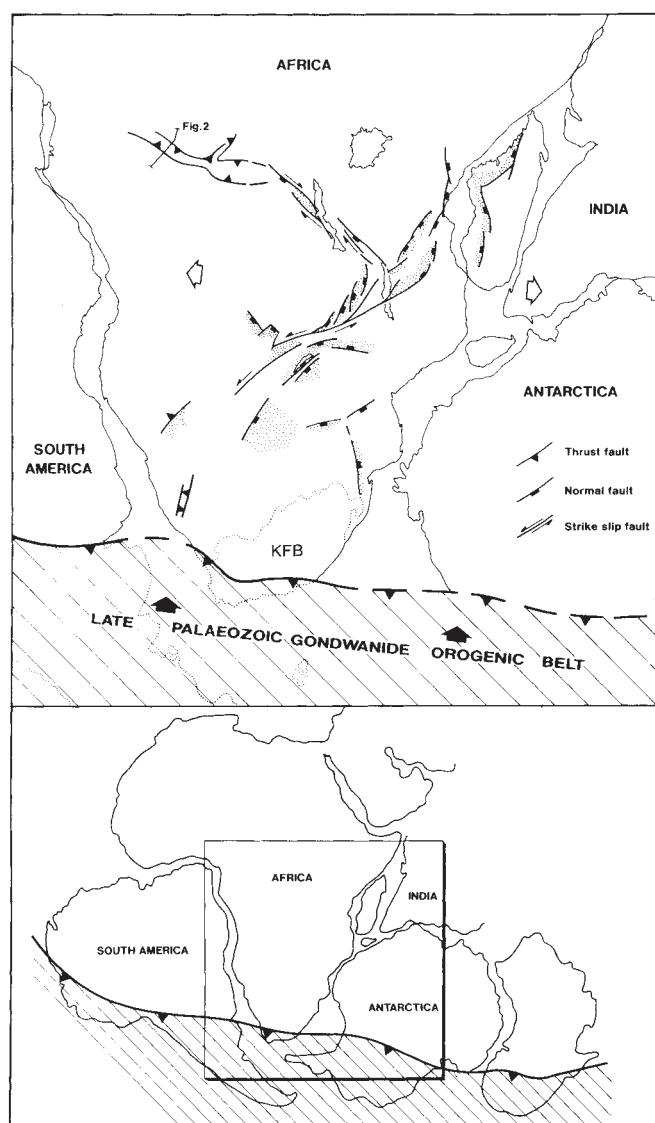


FIG. 4 Gondwana reconstruction showing the major Triassic structures of central Africa and adjacent Madagascar. Note the mixture of normal, strike-slip and thrust structures, and the regional setting of the cross-section in Fig. 2. Stippling shows areas of Permo-Triassic rift sedimentation and cross-hatching the area of the internal zones of the Gondwana orogenic belt. KFB, Karoo foreland basin.

Equal mating success among male reproductive strategies in a marine isopod

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THREE genetically discrete male morphs coexist in *Paracerceis sculpta*, a Gulf of California marine isopod¹⁻⁵. The large α males defend harems within intertidal sponges, the smaller β males mimic female behaviour and morphology, and the tiny γ males invade and sequester themselves within large harems. If selection is responsible for maintaining this polymorphism, then the mean fitness of each male morph must be equal over time⁶⁻⁹. Here we report that average reproductive success is equivalent among the three male morphs in monthly population samples collected over two years. We have investigated the total opportunity for sexual selection within and among morphs, and find that <0.10% of the total opportunity for sexual selection occurs among morphs. Furthermore, alleles responsible for the expression of this polymorphism conform to the Hardy-Weinberg equilibrium, indicating the absence of differential natural selection among morphs. Conditions necessary for stable coexistence of three alternative male reproductive strategies seem to exist in nature.

When isolated with females, the three male morphs (Fig. 1) do not differ in their ability to sire young successfully^{1,2}. Male fertilization success does depend, however, on the relative density of males and females within spongocoels. Experiments using genetic markers show that α males sire the majority of offspring when defending spongocoels containing a single female and one β , or one γ male² (S.M.S. and C. Sassaman,

TABLE 1 Rules for assigning male mating success

Case	Rule
1	α male alone; sires all
2	β male alone; sires all
3	γ male alone; sires all
4	2 α males; each sires half
5	2 γ males; each sires half
6	3 α males; each sires one third
7	1 α + 1 β male; β sires 0.60, α sires 0.40*†
8	1 α + 1 β + 4 γ males; β sires 0.60, α sires none, the remaining progeny are divided among γ males
9	1 α + 2 β + 3 γ males; 0.60 of the progeny are divided between the β males and 0.40 among the γ males, the α male sires none
10	1 α + 1 γ male; γ sires 0.08, α male sires 0.92*†
11	1 α + 2 γ males; each γ follows rule 10‡
12	1 α + 3 γ males; each γ sires 0.08, α male sires the rest
13	2 α + 1 γ ; γ follows rule 10‡, the remaining offspring are divided between the α males
14	2 α + 3 γ males; offspring are evenly divided among the γ males

* Laboratory tests².

† Field data³.

‡ Linear regression based on refs 2 and 3: $y = -0.047 + 0.113x$.

unpublished data). When more than one female is present, β males sire ~60% of all offspring within a spongocoel regardless of female density, although there is considerable variation about this average. The reproductive success of γ males increases linearly with harem size. Paternity rules derived from these data for other combinations of males and females within spongocoels are described in Table 1.

Using these rules, and the observed joint distribution of 825 females and 555 males collected in monthly samples from a natural population over two years (Table 2), we calculated the mean and variance of male mating success and the opportunity



FIG. 1 The three male morphs in *Paracerceis sculpta*. Left to right, γ male, β male, α male.

TABLE 2 Distribution of α , β and γ males and females observed in spongocoels

Harem size	Mating success rule													
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
	1α+			1α+			2α+			2α+			2α+	
	1α	1β	1γ	2α	2γ	3α	1β	1β+4γ	2β+3γ	1γ	2γ	3γ	1γ	3γ
0	121	4	5	1	1	0	2	0	0	5	1	0	0	0
1	101	0	5	2	1	0	4	0	0	8	1	1	0	0
2	55	0	2	2	0	1	4	0	0	7	1	0	0	0
3	33	0	1	1	1	0	2	0	0	3	1	0	1	0
4	22	0	0	2	0	0	0	0	0	6	1	0	1	0
5	17	0	0	0	0	0	1	0	0	2	0	0	0	0
6	6	0	0	1	0	0	0	0	0	2	0	0	0	0
7	0	0	0	2	0	0	0	0	0	0	0	0	0	0
8	2	0	0	0	0	0	0	0	0	0	0	0	0	0
9	1	0	0	1	0	0	0	0	0	0	0	0	0	0
10	1	0	0	0	0	0	0	0	0	0	0	0	0	0
11	1	0	0	0	0	0	0	0	0	1	0	0	0	0
12	0	0	0	0	0	0	0	0	0	0	0	0	0	1
13	0	0	0	0	0	0	0	0	0	1	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0	0	0	0	0
15	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Σ	360	4	13	12	3	1	13	1	1	35	5	1	2	1

Sponges containing isopod breeding aggregations were collected monthly between October 1983 and November 1985 from permanent tide pools in the midintertidal zone, 1.5 km SW of Puerto Peñasco, Sonora, Mexico. Within 15 randomly selected 0.25-m plots along a 100-m transect, all sponges were removed and spongocoels were examined for isopods which were then separated by sex and male type.

for sexual selection¹⁰⁻¹². The opportunity for sexual selection (I) measures the sex difference in the variance of relative reproductive success¹⁰⁻¹². In many species, the variance in male reproductive success is greater than that in females because of variations among males in the numbers of mates. This sex difference in the variance in fitness permits total selection on males to be stronger than on females, and allows the evolution of sexual dimorphism.

We partitioned the observed variation in relative male mating success into two components, that within and that among males. The within-male component measures the strength of sexual selection acting on males within the same strategy. The among-male component measures the strength of sexual selection acting among the three different male reproductive strategies. When the different male reproductive strategies have equal average mating success, there is no opportunity for sexual selection among the male types.

We found that average mating success is statistically equivalent among the three male morphs, and that <0.10% of the total opportunity for sexual selection is acting among the male strategies (Table 3). Even this small fraction is probably an overestimate because the equal apportionment of paternity

within spongocoels, for example by rules 4, 5 and 6 in Table 1, reduces the within-male variance in reproductive success.

Genetic experiments indicate that male morphology in this species is principally influenced by a single, autosomal locus (S.M.S. and C. Sassaman, unpublished data). The ' β allele' at this locus seems to be dominant to both the ' α allele' and the ' γ allele', and the ' γ allele' seems to be dominant to the ' α allele'. Morphological differences among males result from the influence these alleles exert on male growth and maturation rates. Gamma males mature most rapidly ($\bar{x} \pm \text{s.e.} = 57.62 \pm 3.88$ days; $N = 21$); β males mature at an intermediate rate ($\bar{x} \pm \text{s.e.} = 62.33 \pm 6.48$ days; $N = 9$); α males mature most slowly ($\bar{x} \pm \text{s.e.} = 83.61 \pm 3.47$ days; $N = 18$). Differences in maturation rate (1/days to maturity) among the morphs could influence their relative contributions to the population. However, reproductive tenure (age at death minus age at maturity) varies inversely with maturation rate (Fig. 2). Life history differences that influence the relative contributions of α , β and γ males to the population seem, therefore, to cancel.

As a test of this hypothesis we calculated the expected population frequencies for α , β and γ males, assuming the three alleles at the male locus are in Hardy-Weinberg equilibrium. Morph

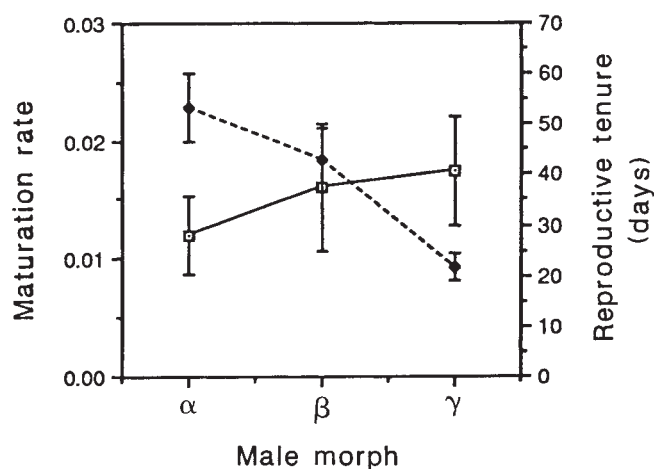


FIG. 2 The relationship between maturation rate (solid line; $1/(\text{days from birth to maturity})$; $y = 1.87 - 0.19x$, $R^2 = 0.38$, $P < 0.001$) and reproductive tenure (dashed line; age at death minus age at maturity, in days; $y = 0.01 + 0.02x$, $R^2 = 0.36$, $P < 0.001$) for α ($N = 18$), β ($N = 9$) and γ males ($N = 21$). Bars represent one standard error.

TABLE 3 Mean and variance of male mating success and the opportunity for sexual selection

Male type	Mean ($\pm$ s.e.) of number of mates	N
α males	1.51 $\pm$ 0.08	452
β males	1.35 $\pm$ 0.44	20
γ males	1.37 $\pm$ 0.23	83
Grand mean	1.49 mates per male	
The opportunity for sexual selection, I		
Variance among-types	0.003	
Variance within-types	3.075	
$I_{\text{total}} = (\text{Total variance})/(\text{Grand mean})^2 = 1.39$		
$I_{\text{among}} = (\text{Variance among-types})/(\text{Grand mean})^2 = 0.001$		

frequencies (Table 1) are typical for this population^{1,2,4,5} (α males, 0.814; β males, 0.036; γ males, 0.150; $N = 555$). As α males are recessive homozygotes, the frequency of the α allele is $(0.814)^{1/2} = 0.902$. The rarity of β males suggests that nearly all β males in the population are heterozygotes. Thus, to a first approximation, the frequency of the β allele is $1/2(0.036) = 0.018$. Because the sum of the three allele frequencies must equal unity, the frequency of the γ allele is 0.080. Therefore, the expected population frequencies for α males (α^2), β males ($2\alpha\beta + 2\beta\gamma$) and γ males ($\gamma^2 + 2\alpha\gamma$) are 0.814, 0.035 and 0.151. The observed frequency of the γ -male morph (0.150) conforms to the theoretical expectation ($\gamma^2 + 2\alpha\gamma = 0.151$).

Most polymorphisms in male mating behaviour seem to be 'condition dependent', in that males adjust their mating tactics to fit local environmental circumstances^{6,7,13-27}. The presumed ancestral condition for male alternative strategies⁹, however, and the model for the evolution of polymorphism in general, applies to male mating strategies that are genetically distinct^{3,8,28-30}. Male polymorphism in *P. sculpta* provides such a model.

We have shown that average mating success is statistically equivalent among the three male morphs and that a tiny fraction of the total opportunity for sexual selection acts among the male strategies. Moreover, alleles responsible for the expression of male phenotype in this species conform to Hardy-Weinberg equilibrium. We conclude therefore that there is no significant selection favouring one male reproductive strategy, and that the necessary conditions for maintaining a polymorphism in male mating behaviour exist in this natural population. $\square$

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Cloning of the gene for a human dopamine D₄ receptor with high affinity for the antipsychotic clozapine

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DOPAMINE receptors belong to the family of G protein-coupled receptors. On the basis of the homology between these receptors, three different dopamine receptors (D₁, D₂, D₃) have been cloned¹⁻⁷. Dopamine receptors are primary targets for drugs used in the treatment of psychomotor disorders such as Parkinson's disease and schizophrenia^{8,9}. In the management of socially withdrawn and treatment-resistant schizophrenics, clozapine¹⁰ is one of the most favoured antipsychotics because it does not cause tardive dyskinesia¹¹. Clozapine, however, has dissociation constants for binding to D₂ and D₃ that are 4 to 30 times the therapeutic free concentration of clozapine in plasma water^{12,13}. This observation suggests the existence of other types of dopamine receptors which are more sensitive to clozapine. Here we report the cloning of a gene that encodes such a receptor (D₄). The D₄ receptor gene has high homology to the human dopamine D₂ and D₃ receptor genes. The pharmacological characteristics of this receptor resembles that of the D₂ and D₃ receptors, but its affinity for clozapine is one order of magnitude higher. Recognition and characterization of this clozapine neuroleptic site may prove useful in the design of new types of drugs.

Various tissues and cell lines were screened for the presence of dopamine-like receptors by northern blot analysis. RNA isolated from human neuroblastoma SK-N-MC cells (ATCC) was hybridized to a radiolabelled 300-base-pair (bp) *Bst*YI-*Bgl*II fragment of the rat D₂ receptor¹ which encodes the putative transmembrane domains VI and VII. Under low-stringency conditions, several bands were detected on the northern blots (data not shown), but under high-stringency conditions none of the bands hybridized with the probe. This suggested that the cell line contained other receptors homologous but not identical to the D₂ receptor.

A complementary DNA library of the neuroblastoma SK-N-MC was constructed in the vector λ ZAPII. About 500,000 independent clones were screened under low-stringency conditions, using the 300-bp *Bst*YI-*Bgl*II fragment and a 1.6-kilobase (kb) *Bam*HI-*Bgl*II fragment containing the complete coding sequence of the rat D₂ receptor¹. Several different clones were identified by this procedure. One of the clones, called D₂10S, contained a 780-bp *Eco*RI-*Xho*I insert which has a high degree of homology to sequences encoding the putative transmembrane

TABLE 1 Drug dissociation constants (K_i)

	D2 K_i (nM)	Ref.	D4 K_i (nM)	$K_{i,D2}$
				$K_{i,D4}$
Antagonists:				
Butaclamol-(+)	0.8 H	p	40	0.02
Chlorpromazine	2.8 R	7	37	0.08
	1.5 H	30		0.04
Clozapine	-130 T	27	9	14.4
	56 R	7		6.2
	138 H	p		15.3
Eticlopride	0.09 T	28	2.1	0.04
Fluphenazine	0.5 T	28	46	0.01
Haloperidol	0.5 R	7	5.1	0.10
	0.8 R	31		0.16
	1 H	2		0.2
Ketanserin	192 T	32	148	1.3
Octoclotheptin-S	1.5 T	29	0.6	2.5
Octoclotheptin-R	13.5 T	29	1.6	8.4
Pimozide	2.4 R	7	43	0.06
Raclopride	1.8 R	7	237	0.008
	10.5 H	p		0.04
*Raclopride	3.2 H	30		0.01
Remoxipride	~300 T	27	3,690	0.08
SCH 23390	913 H	2	3,560	0.26
Spiperone	0.069 R	7	0.05	1.38
	0.053 H	2		1.06
*Spiperone	0.05 H	3	0.08	0.63
	0.03 H	p		0.34
Sulpiride-S	9.2 R	7	52	0.18
	4.8 R	31		0.09
	46 H	30		0.89
	31 H	p		0.60
Thioproperazine	0.21 R	7	53	0.004
Thioridazine	3.3 R	7	12	0.28
Trifluoperazine	1.2 T	28	3	0.40
YM-09151-2	0.06 T	28	0.09	0.67
*YM-09151-2	0.09 H	p		1.00
Agonists:				
ADTN-(±)	Hi: 1.7 T	28	Hi: 24	
Apomorphine	Hi: ~2 T	28	Hi: 4.1	
	24 R	7		
Bromocriptine	5.3 R	7	340	
	12.6 H	p		
Dopamine	Hi: 7.5 T	28	Hi: 28	
	Hi: 2.8 R	31		
	474 R	7		
Dopamine + G	17,000 R	7	450	
Ergocriptine-β	Hi: 0.4 T	28	109	
Fenoldopam	Hi: 2.8 T	28	321	
N-0437	Hi: 0.7 T	28	122	
(-)-Noradrenaline	~6,000 T	28	1,760	
NPA	Hi: 0.4 T	28	6.5	
PHNO-(+)	Hi: 1.2 T	28	79	
Quinpirole(-)	576 R	7		
	Hi: 4.8 T	28	46	
Serotonin	~10,000 T	28	4,180	
SKF 38393	Hi: 157 T	28	1,800	
	9,560 R	7		

Varying concentrations of dopamine agonists and antagonists (10^{-14} – 10^{-4} M) were used to inhibit [3 H]spiperone (150–200 pM) binding to membranes prepared from the COS-7 cells transfected with a 3.9-kb cDNA–gene fusion construct, or mouse Ltk⁻ cells expressing the human dopamine $D_{2(long)}$ receptor². Dissociation constants were obtained by computer assisted analysis (LIGAND) as described³ and are the average of at least two separate experiments. Abbreviations: p, present study, using human $D_{2(long)}$ expressed stably in mouse Ltk⁻ cells; H, human $D_{2(long)}$; R, rat $D_{2(long)}$; T, K_i in canine striatum or pig anterior pituitary tissue homogenate; *, tritium; ADTN-(±), (±)-6,7-dihydroxy-2-aminotetralin; G, Gpp[NH]p; NPA, *N*-propylnorapomorphine; N-0437, 2-(*N*-propyl-*N*-thienylethylamino)-5-hydroxytetralin-HCl; quinpirole(-), LY171555. Clozapine and its congeners are boxed.

domains VI and VII of the D_2 receptor (Fig. 1b). The complete nucleotide sequence contains an open reading frame that could encode a polypeptide with homology to the transmembrane domains V (46%), VI (46%) and VII (78%) of the human D_2 receptor² (Fig. 1b and c).

The genomic clone D_{210G} was identified in a human genomic library in EMBL3 (Clontech) by screening with clone D_{210S} under high-stringency conditions (Fig. 1a and b). Southern blot and sequence analysis indicated that the clone contained a 5-kb *SalI*–*PstI* fragment containing the entire gene of D_{210S} . There is a potential initiation codon¹⁴ 590 bp downstream of the *SalI* site followed by an open reading frame that encodes an amino-acid sequence with homology to transmembrane domains I (36%) and II (63%) of the D_2 receptor. This sequence is interrupted 185 bp downstream of the initiation codon by a large putative intron of about 2 kb. The remainder of the gene product showed homology to the transmembrane domains III (68%), IV (37%), V (46%), VI (46%) and VII (78%) of the D_2 receptor. The overall homology of this putative dopamine receptor to the D_1 , D_2 and D_3 receptors is 28%, 41% and 39%, respectively (Fig. 1c).

Potential splice junction donor and acceptor sites¹⁵ were found in the sequences encoding the transmembrane domains II, III and VI (refs 2, 3, 7) (Fig. 1b). These splice sites were at an equivalent position to those in the D_2 and D_3 receptor genes^{2,3,7} (Fig. 1a). The genomic clone D_{210G} is identical to the corresponding region of the partial cDNA clone D_{210S} , but is interrupted between the regions encoding the transmembrane domains V and VI by an intron of 240 bp (intron 3) and within the region encoding transmembrane domain VI by an additional intron of 92 bp (Fig. 1b). Intron 3 has an unusual intron–exon configuration in which the conventional flanking splice-junction donor and acceptor sites¹⁵ are missing. The intron junction sites are part of a 52-bp repeat, but this sequence is present only once in the cDNA (see Fig. 1b, overlined sequence). This novel configuration of intron 3 does not allow a precise placement of the splice junction sites.

Southern blot analysis of human genomic DNA, digested with several restriction endonucleases, indicated that the isolated clone coded for a single-copy gene. Using a restriction endonuclease fragment length polymorphism (RFLP) identified with this clone, the gene has been located at the tip of the short arm of chromosome 11 (J. L. Kennedy, personal communication). Attempts to obtain the full-length human cDNA clone either by screening several cDNA libraries or by polymerase chain reaction were unsuccessful.

The amino-acid sequence deduced from the genomic and cDNA sequences indicates that this gene encodes a protein of 387 amino acids with a relative molecular mass (M_r) of 41,000. A hydrophobicity plot of the protein sequence suggests that there are seven transmembrane domains¹⁶ (data not shown). These are the same regions that are homologous to regions in the human D_2 receptor and other receptors belonging to the family of G protein-coupled receptors^{1–7,16} (Fig. 1c). There is a potential *N*-linked glycosylation site¹⁷ two amino acids downstream from the initiation methionine. The Asp 80 and Asp 115 in the D_4 receptor, conserved within the family of catecholaminergic receptors, are postulated to act as counterions in catecholamine binding¹⁸. Ser 197 and Ser 200, which have been suggested to interact with the catechol hydroxyl groups,¹⁹ are also conserved within the family of catecholaminergic receptors. Several consensus sites for possible phosphorylation by protein kinase C and protein kinase A exist in the third cytoplasmic loop^{20,21}. Cys 387, which could serve as a substrate for palmitoylation, is conserved in most of the G protein-coupled receptors²². The short carboxy tail, which terminates, as in the D_2 and D_3 receptor, at Cys 387 (refs 1–3, 7), and the relatively large third cytoplasmic loop, are features observed in most receptors which interact with G_i .

We next determined the pharmacological characteristics of this dopamine-like receptor gene. A 4.3-kb *ApaI*–*PstI* fragment

[illegible]

acgttaattaaacaaattccttcctccaaactcagctgtgaaggctcctggg-3'

↑
(a)₁₈

TRANSMEMBRANE DOMAIN 1

A	G	G	A	A	L	V	G	G	V	L	G	V	L	A	G	S	L	V	G	S	V	R	E	R	A	L	Q	T	P	T					
R	P	H	N	Y	A	T	L	L	T	L	L	A	I	V	F	G	N	V	L	G	M	A	V	S	R	E	K	A	L	Q	T				
R	P	H	N	Y	A	T	L	L	T	L	L	A	I	V	F	G	N	V	L	G	M	A	V	S	R	E	K	A	L	Q	T				
R	P	H	N	Y	A	T	L	L	T	L	L	A	I	V	F	G	N	V	L	G	M	A	V	S	R	E	K	A	L	Q	T				
F	S	V	R	I	L	T	A	C	F	L	S	L	L	T	S	L	L	G	N	T	L	V	C	A	A	V	I	R	F	H	L	R	S	K	V
F	S	V	R	I	L	T	A	C	F	L	S	L	L	T	S	L	L	G	N	T	L	V	C	A	A	V	I	R	F	H	L	R	S	K	V

TRANSMEMBRANE DOMAIN 3

M	A	L	D	V	M	L	C	T	A	S	I	F	N	L	C	A	I	S	V	D	R	F	V	A	V	A	V	P	L	R	Y	N	R	-	Q	G	G	S		
V	T	L	D	V	M	M	C	T	A	S	I	F	N	L	N	C	A	I	S	I	D	R	Y	T	A	V	A	M	P	M	L	Y	N	T	R	Y	-	S	S	S
V	T	L	D	V	M	M	C	T	A	S	I	F	N	L	N	C	A	I	S	I	D	R	Y	T	A	V	A	M	P	M	L	Y	N	T	R	Y	-	S	S	S
V	T	L	D	V	M	M	C	T	A	S	I	F	N	L	N	C	A	I	S	I	D	R	Y	T	A	V	A	M	P	M	L	Y	N	T	R	Y	-	S	S	S
V	A	F	I	R	T	S	T	S	T	S	I	F	N	L	N	C	V	I	S	V	D	R	F	V	A	V	A	M	P	F	Q	Y	E	-	R	K	M	T		
V	A	F	I	R	T	S	T	S	I	F	N	L	N	C	V	I	S	V	D	R	F	V	A	V	A	M	P	F	Q	Y	E	-	R	K	M	T				

TRANSMEMBRANE DOMAIN S

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-----AVCRLEDRDYVYSSVCSPFLPCPLMLLLYWATF  
-----NECITANPAFYVYSSIVSFPVFPIVTLLVIKITY  
-----NECITANPAFYVYSSIVSFPVPFPIVTLLVIKITY  
AETIDNCGOSGDSALSSSSVYIVAFVIVSVIY  
ERTEEDNCDDLS--RTYAISSSLSPYIPVAIMVTTSTSY
```

PAPRLPQDPC**EP**DCAPPAPGLP**EP**DP**EP**GSNCAPPDAVRAAL
 KLCTVI**IK**MSN**IG**SFPVNNRRYDAARAQ**EL**EW**EL**MSSTSPPE
 KLCTVI**IK**MSN**IG**SFPVNNRRMDAARAQ**EL**EW**EL**MSSTSPPE
 SI¹RAHF¹LS¹DA¹T**G**QMEH¹EDKQY**EP****EP****EP**QDPLLSHLQPPSPGQ


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- - - - - R R R A K - I T G R R R K A M R
- P K I A K I F E I Q T M P N G K T R T S L K T M S R R - K L S Q O Q K E K K A T Q
N P R I A K F F E I Q T M P N G K T R T S L K T M S R R - K L S Q O Q K E K K A T Q
A P K L S - L - E V R K L S N G R L S T L R L G P L O P R G V P L R E K K A T Q
- - - - - T T T G N G K P V E C S Q P E S S - F K M S F K R E T K V L K
- - - - - T T A G N G N P V E C A Q S E S S - F K M S F K R E T K V L K

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V T W L G Y V N S A L N P V I Y T V F N A E F R N V F R K A F R A C G 387
 F T W L G Y V N S A L N P I I Y T T F N I E F R - - - K A F K I L H C 443
 F T W L G Y V N S A L N P I I Y T T F N I E F R - - - K A F K I L H C 444
 F T W L G Y V N S A L N P I I Y T T F N I E F R - - - K A F K I L S C 445
 F V M F G W A N S S L N P I I Y A F N A O F O - - - K A F S T L L G C Y R L C
 F V M F G W A N S S L N P I I Y A F N A O F O - - - K A F S T L L G C Y R L C

VGSSSEDLKKEEAAGI ARPLEKLSPALSVI LDYDTDVSLEKI
VGSSSEDLKKEEAAGGI AKPLEKLSPALSVI LDYDTDVSLEKI

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FIG. 1 *a*, Restriction map of the genomic human dopamine D₄ receptor clone and alignment with the genomic intron-exon organization of the human dopamine D₂ receptor². Relevant restriction endonuclease sites in the D₄ receptor are indicated. The *Sal*I site is part of the cloning site in EMBL3. The proposed coding regions are boxed and numbered in Arabic numerals. Perfect matches of proposed intron-exon junction sites are indicated by connecting broken lines between the receptor clones. The black boxes labelled by Roman numerals indicate the putative transmembrane domains. *b*, Nucleotide and deduced amino-acid sequence of the human dopamine D₄ receptor gene and cDNA. The putative coding sequence is in capitals (noncoding sequence is in italics) and deduced amino-acid sequence is shown below the nucleotide sequence. Numbering of the putative coding sequence begins with the first methionine of the open reading frame. The beginning of the coding sequence corresponding to the cDNA clone is indicated by an arrow at nucleotide 517. The beginning of the poly(A) tail in the cDNA clone is indicated by an arrow 101 nucleotides downstream from the stop codon. A 52-bp repeat bordering both sites of the third intron is underlined. The *Pst*I restriction endonuclease site used in the construction of the hybrid between gene and cDNA is underlined. ▲, Potential *N*-glycosylation site; ■, putative protein kinase A phosphorylation site; □, putative protein kinase C phosphorylation site. *c*, Alignment of the putative amino-acid sequence (single-letter code) of the human D₄ receptor with the human and rat D₁ and D₂, and rat D₃ receptors¹⁻⁷. Amino acids conserved between

the D₄ and the other dopamine receptors are boxed. The putative transmembrane domains are indicated by bars and labelled in Arabic numerals.

METHODS. Double-stranded cDNA was synthesized from poly(A)⁺ mRNA isolated from the human neuroblastoma SK-N-MC. The cDNA was directionally cloned into the *Eco*RI and *Xho*I sites of λ ZAPII (Stratagene). The library was transferred to colony plaque screen filters (NEN) and prehybridized overnight at 37 °C in 25% formamide, 0.2% polyvinylpyrrolidone (*M_w* 40,000), 0.2% Ficoll (*M_w* 400,000), 0.05 M Tris buffer, pH 7.5, 1 M NaCl, 0.1% tetrasodium pyrophosphate, 1% SDS and denatured salmon sperm DNA (100 μ g ml⁻¹). The library was hybridized for 30 h under similar conditions with ³²P-labelled (10⁶ d.p.m. ml⁻¹, random-primed labelling system, Boehringer Mannheim) 1.6-kb *Bam*HI-*Bgl*II and 300-bp *Bst*YI-*Bgl*II fragments of the D₂ receptor. Filters were washed at 55 °C in 2 × SSC, 1% SDS. The clone D₂10S was isolated and sequenced. Clone D₂10S was ³²P-labelled by random-primed synthesis and used to screen a genomic library in EMBL3 (Clontech) under similar conditions as described above but using 50% formamide. The filters were washed at 65 °C in 0.1 × SSC, 0.1% SDS. The clone D₂10G was isolated and analysed by restriction endonuclease and southern blot analysis. A 1.3-kb and 2.6-kb *Pst*I-*Pst*I fragment, and an overlapping 2.0-kb *Sal*I-*Eco*RI fragment were subcloned into pBluescript (SK⁻) (Stratagene). The subcloned fragments were sequenced using the dideoxy chain termination method in combination with 7-deaza-dGTP and Sequenase V2.0 (USB).

containing the entire gene was subcloned into the expression vector pCD-PS (ref. 23). Low levels of saturable [³H]spiperone binding were detected in COS-7 cells transfected²⁴ transiently with this plasmid construct. The [³H]spiperone binding could be inhibited in a concentration-dependent manner by several dopamine agonists and antagonists, of which dopamine and clozapine demonstrated dissociation constants of about 20 nM and 10 nM, respectively. The levels of expression were, however, not sufficient for adequate pharmacological characterization.

In general, conventional introns have been shown to splice out efficiently from genes introduced into COS-7 cells^{25,26}. However, the absence of conventional splice donor and acceptor sites for the third intron might affect the efficiency of splicing of

this intron in COS-7 cells. As we were unable to obtain a full-length cDNA clone, a hybrid gene-cDNA was constructed in which the genomic sequence downstream from the *Pst*I site (Fig. 1*b*, underlined sequence) was replaced with the cDNA sequence. This 3.9-kb hybrid gene-cDNA fragment was subcloned into the expression vector pCD-PS (ref. 23) COS-7 cells transfected with this construct displayed saturable [³H]spiperone binding (200–300 fmol mg⁻¹ protein) with a similar dissociation affinity constant (70 pM) as that of the cloned D₂ receptor (Fig. 2*a*). Dopaminergic agonists and antagonists inhibited [³H]spiperone binding to these cells in a concentration-dependent and stereoselective manner with an appropriate pharmacological profile for dopamine receptors

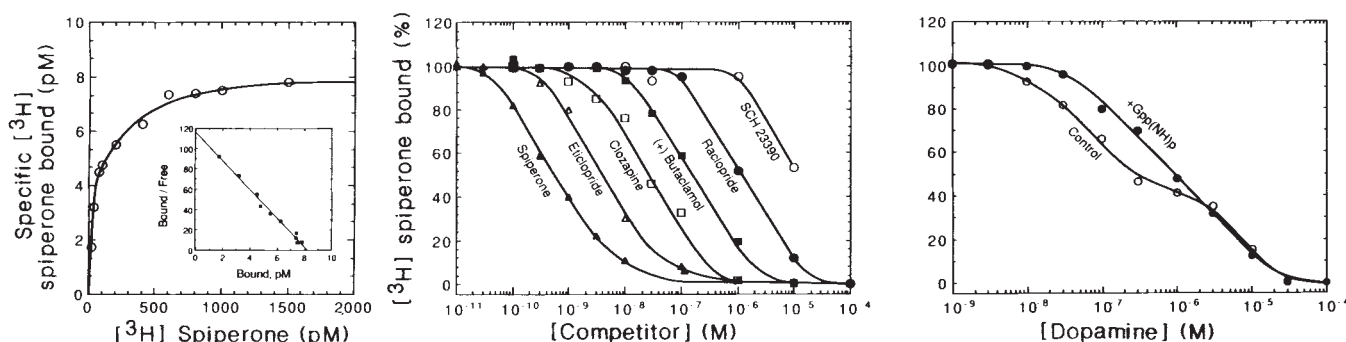


FIG. 2 Binding of [³H]spiperone to transfected COS-7 cell membranes. *a*, Saturation isotherms of the specific binding of [³H]spiperone to membranes from COS-7 cells expressing the cloned human dopamine D₄ receptor. The results shown are representative of two independent experiments each conducted in duplicate. Inset, Scatchard plot of the same data. Estimated *B_{max}* (maximal receptor density; ~260 fmol mg⁻¹ protein) and dissociation constants (*K_d*; 70 pM) values were obtained by LIGAND, as previously described⁵. *b*, Pharmacological specificity of [³H]spiperone binding to transfected COS-7 cells. Representative curves are shown for the concentration-dependent inhibition of [³H]spiperone binding by various dopamine agonists and antagonists. Data were analysed by LIGAND and the results shown are the means of duplicate determinations. Estimated inhibition constants (*K_i*) are listed in Table 1 along with the *K_i* values obtained on the human D₂(long) receptor stably expressed in mouse Ltk⁻ cells². *c*, Modulation of dopamine interaction with the human dopamine D₄ receptor by Gpp(NH)p.

METHODS. A 3.9-kb gene-cDNA hybrid was subcloned into the expression vector pCD-PS (ref. 23). This plasmid construct was introduced into COS-7 cells by calcium phosphate-mediated transfection as described²⁴. Cells were

collected and homogenized (Teflon pestle) in 50 mM Tris-HCl (pH 7.4 at 4 °C) buffer containing 5 mM EDTA, 1.5 mM CaCl₂, 5 mM MgCl₂, 5 mM KCl and 120 mM NaCl. Homogenates were centrifuged for 15 min at 39,000 *g*, and the resulting pellets resuspended in buffer at a concentration of 150–250 μ g ml⁻¹. For saturation experiments, 0.25-ml aliquots of tissue homogenate were incubated in duplicate with increasing concentrations of [³H]spiperone (70.3 Ci mmol⁻¹; 10–3,000 pM final concentration) for 120 min at 22 °C in a total volume of 1 ml. For competition binding experiments, assays were initiated by the addition of 0.25 ml membrane and incubated in duplicate with the indicated concentrations of competing ligands (10⁻¹⁴–10⁻³ M) and [³H]spiperone (150–300 pM) in either the absence or presence of 200 μ M Gpp(NH)p, where indicated, for 120 min at 22 °C. Assays were terminated by rapid filtration through a Titertek cell harvester and filters then monitored for tritium as described⁵. For all experiments, specific [³H]spiperone binding was defined as that inhibited by 10 μ M (–)sulpiride. Both saturation and competition binding data were analysed by the non-linear least-square curve-fitting program LIGAND run on a Digital Micro-PDP-11 as described⁵.

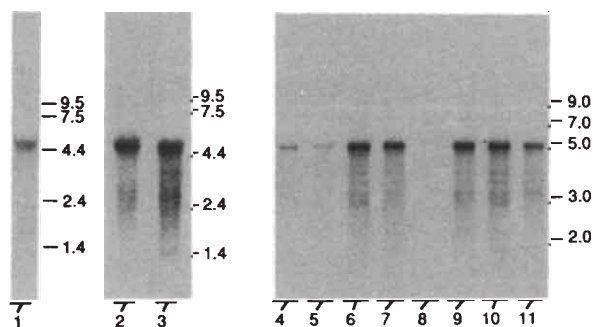


FIG. 3 Northern blot analysis of D_4 mRNA in the human neuroblastoma SK-N-MC, and rat and monkey brain areas. Northern blot analysis of poly(A)⁺mRNA (SK-N-MC, 10 μ g; rat brain areas, 7 μ g; monkey brain areas, 4 μ g) was performed as previously described³ but probed with random-primed ³²P-labelled D_2 10S (SK-N-MC cells and rat brain areas) or a ³²P-labelled 417-bp fragment of D_2 10G encoding transmembrane domains VI and VII starting at the 5' site of the fourth intron which was made using the polymerase chain reaction. After hybridization the blots containing mRNA from the SK-N-MC, rat brain, and monkey brain were exposed for 3 days at -80°C to X-ray film with an intensifying screen. Numbers on the sides of the panels are size markers in kb. Lanes 1, SK-N-MC; 2, rat striatum; 3, rat brain area comprising hypothalamus, mesencephalon, medulla; monkey brain areas: 4, olfactory tubercle; 5, hippocampus; 6, medulla; 7, amygdala; 8, cerebellum; 9, midbrain; 10, frontal cortex; 11, striatum.

(Fig. 2b). COS-7 cells expressing the D_4 receptor also produced a guanine nucleotide-sensitive high-affinity form of the receptor on binding of dopamine agonists (Fig. 2b, and Table 1).

Most of the agonists and antagonists tested had similar or lower affinities for this receptor (D_4) than for the D_2 and D_3 receptors. But the atypical neuroleptic clozapine and its congener octoclotheptin had a higher affinity for the D_4 receptor. Interestingly, the dissociation constant of about 9 nM for clozapine at the D_4 receptor matches the plasma water concentration of clozapine under therapeutic conditions (derived from its concentration in plasma¹², of which 5% is free¹³, in patients receiving 2–4 mg kg⁻¹ day⁻¹ doses). This suggests that the D_4 receptor may be the primary dopamine receptor that mediates the antipsychotic action of clozapine.

The distribution of the D_4 messenger RNA in the brain was examined by northern blot analysis (Fig. 3). This showed the presence of a mRNA of about 5.3 kb in the human neuroblastoma SK-N-MC and in several regions of the brains of rat and monkey. Based on these analyses, we estimate that, in regions where these mRNAs are relatively well expressed, concentrations of D_4 and D_3 mRNA are similar, but those of D_2 mRNA seem to be 1–2 orders of magnitude higher. Relatively high levels for the D_4 mRNA were observed in the monkey frontal cortex, midbrain area, amygdala and medulla, but lower levels were detected in the basal ganglia. This distribution profile differs from the D_2 and D_3 mRNA (refs 1, 7) and may partly explain the absence of tardive dyskinesia, rigidity (Parkinsonism) and akinesia during clozapine treatment¹¹.

The identification of a gene coding for a dopamine receptor with high affinity for clozapine is important for our understanding of the clinical effectiveness of this antipsychotic. The use of this antipsychotic, which lacks most of the common side effects of neuroleptics, is nevertheless limited because of the risk of agranulocytosis¹³. The isolation and expression of this receptor gene may help in the design of improved clozapine analogues. □

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Cloning of the gene for a human dopamine D_5 receptor with higher affinity for dopamine than D_1

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DOPAMINE receptors belong to a superfamily of receptors that exert their biological effects through guanine nucleotide-binding (G) proteins. Two main dopamine receptor subtypes have been identified, D_1 and D_2 , which differ in their pharmacological and biochemical characteristics. D_1 stimulates adenylyl cyclase activity, whereas D_2 inhibits it^{1–3}. Both receptors are primary targets for drugs used to treat many psychomotor diseases, including Parkinson's disease and schizophrenia^{4,5}. Whereas the dopamine D_1 receptor has been cloned^{6–9}, biochemical and behavioural data indicate that dopamine D_1 -like receptors exist which either are not linked to adenylyl cyclase or display different pharmacological activities^{10,11}. We report here the cloning of a gene encoding a 477-amino-acid protein with strong homology to the cloned D_1 receptor. The receptor, called D_5 , binds drugs with a pharmacological profile similar to that of the cloned D_1 receptor, but displays a 10-fold higher affinity for the endogenous agonist, dopamine. As with D_1 , the dopamine D_5 receptor stimulates

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TABLE 1 K_i values for [3 H]SCH-23390 binding to D_5 and D_1 receptors expressed in COS-7 cells

Agonists	D_5	D_1	D_1/D_5	Antagonists	D_5	D_1	D_1/D_5
(+)-SKF-82526	15	28	1.86	SCH-23390	0.30	0.35	1.17
SKF-38393	100	150	1.50	SKF-83566	0.40	0.30	0.75
β -Ergocriptine	113	—	—	α -Flupenthixol	8	4	0.50
Dopamine	228	2,340	10.29	Fluphenazine	14	21	1.50
Apomorphine	363	680	1.87	(+)-Butaclamol	27	3	0.11
Bromocriptine	454	672	1.48	SCH-23388	35	41	1.17
SKF-76783	530	645	1.22	Haloperidol	48	27	0.56
(-)-SKF-82526	800	1,818	2.27	Chlorpromazine	133	73	0.55
(+)-ADTN*	909	4,600	5.06	Clozapine	250	141	0.56
Pergolide	918	1,363	1.48	Thioridazine	300	100	0.33
N-0437	986	2,172	2.20	Ketanserin	2,500	190	0.07
NPA*	1,136	1,816	1.59	Spiperone	4,500	220	0.05
Serotonin	3,000	9,690	3.23	Eticlopride	19,000	18,000	0.95
Noradrenaline	12,000	50,000	4.16	(+)-Sulpiride	28,636	20,454	0.71
LY-171555	>20,000	>20,000	—	(-)-Sulpiride	77,270	36,000	0.46

Inhibitory constants (K_i , in nM) of various dopaminergic agonists and antagonists are listed in order of their potency for the human dopamine D_5 receptor. Transfection of COS-7 cells with the dopamine D_5 and D_1 gene⁶ was performed as described in Fig. 2. The dissociation constants were obtained using the computer programme LIGAND. Values represent the means of two or three independent experiments conducted in duplicate and varied by less than 10%.

* ADTN, aminotetralin-6,7-diol; NPA: *N*-propylnorapomorphine.

adenylyl cyclase activity. Northern blot and *in situ* hybridization analyses reveal that the receptor is neuron-specific, localized primarily within limbic regions of the brain; no messenger RNA was detected in kidney, liver, heart or parathyroid gland. The existence of a dopamine D_1 -like receptor with these characteristics had not been predicted and may represent an alternative pathway for dopamine-mediated events and regulation of D_2 receptor activity¹²⁻¹⁴.

Although dopamine receptors have been classically divided into two functional groups, termed D_1 and D_2 , many studies have suggested the existence of several different types of dopamine receptor. Based on the strong nucleotide and amino-acid sequence homology between members of the G-linked receptor family¹⁵, we probed a λ EMBL 3 SP6-T7 human genomic library (Clontech) with a 500-base-pair (bp) *Bgl*II-*Hind*III fragment of DNA encoding transmembrane regions two to five of the human D_1 receptor under low-stringency conditions. Two clones were isolated each containing inserts of about 22 kilobases (kb). Restriction map and Southern blot analysis of one of these clones, RS6, digested with *Xho*I revealed a fragment of about 5 kb (RS7) which was subcloned into pSP73 (Promega). The nucleotide sequence of RS7 was determined and found to contain a putative initiation methionine codon¹⁶ followed by a long open reading frame of 1,431 nucleotides, encoding a 477-amino-acid protein with an estimated relative molecular mass (M_r) of 53,000. Figure 1a and c shows a partial restriction map of RS7 and its nucleotide and deduced amino-acid sequence. As with the dopamine D_1 receptor, but unlike the dopamine D_2 (ref. 17), D_3 (ref. 18) and D_4 (ref. 19) receptors, RS7 does not contain an intron in its coding sequence. To confirm that RS7 had no intron, specific oligonucleotide primers were used to selectively amplify complementary DNA from a λ gt11 human putamen brain library (Clontech) using the polymerase chain reaction. A number of fragments of different size, which together encompassed most of the coding region of the gene (from 40 bp after the initiating methionine to 51 bp before the stop codon), were isolated, subcloned into pSP73 and sequenced (Fig. 1b). The amplified cDNA fragments were identical in sequence to the corresponding part of the gene encoded by RS7.

Hydrophobicity analysis of the amino-acid sequence of RS7 indicates the presence of seven transmembrane domains characteristic of all members of the G protein-coupled superfamily of receptors (data not shown). A comparison of the deduced amino-acid sequence of RS7 with that of other cloned dopamine receptors shows that the greatest similarities are between putative transmembrane domains, particularly in the case of the

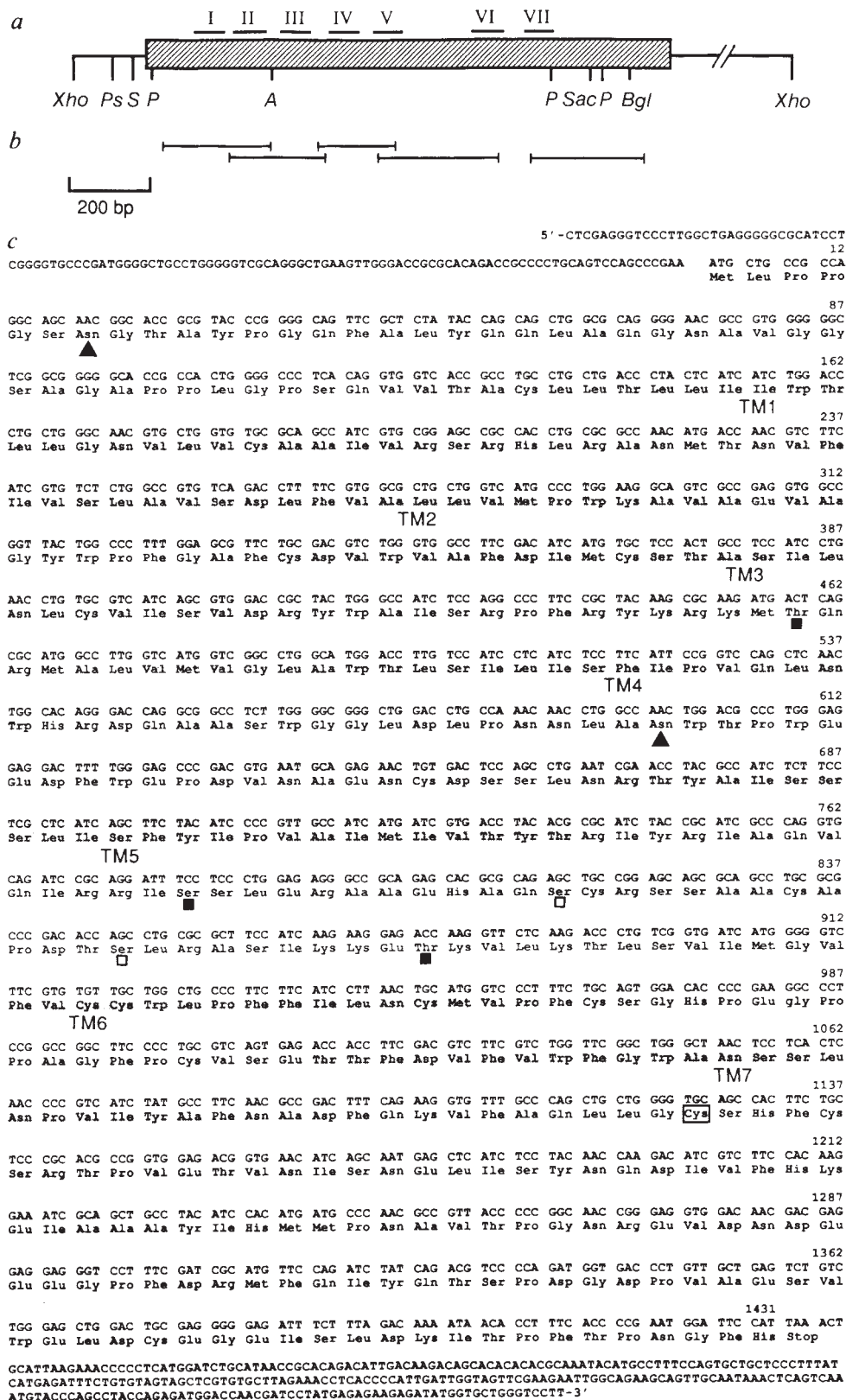
dopamine D_1 receptor where the homology is 80%. Figure 1d shows the amino-acid homology between the human RS7 receptor and both the human and rat dopamine D_1 and D_2 (ref. 20) receptors, and the rat D_3 receptor. The degree of homology between RS7 and other dopamine receptors is 30% for D_2 and D_3 , 50% for D_1 , and about the same between RS7 and other catecholaminergic receptors.

The proposed N terminus contains a consensus site for *N*-linked glycosylation and, as with the dopamine D_1 receptor, there is an additional site in the second extracellular loop. There are several putative phosphorylation sites for protein kinase C and cyclic AMP-dependent protein kinase in the second and third intracellular loops (Fig. 1c). The deduced amino-acid sequence of RS7 reveals a conserved Asp (at position 120) in the third cytoplasmic loop, as well as Ser 230 and Ser 233 in the fifth transmembrane region, which are conserved in all catecholamine receptors and may form part of the proposed catecholamine binding site²¹. Also conserved in RS7 is Cys 375, which is located in the C terminus and could be a palmitoylation site²². Furthermore, the size and sequence of the third cytoplasmic loop of RS7, as well as the size of the C-terminal tail, are similar to that of both the dopamine D_1 and β -adrenergic receptors¹⁵, suggesting that this new receptor might be coupled to G_s and stimulate adenylyl cyclase. On the basis of these structural characteristics, RS7 is thought to encode a novel dopamine receptor with D_1 -like features.

To identify the pharmacological profile of this clone, the 5-kb *Xho*I fragment was subcloned into the eukaryotic expression vector pCD-PS (ref. 23) and transiently expressed in COS-7 cells. The gene product of pCD-RS7 bound the specific dopamine D_1 antagonist [3 H]SCH-23390 in a dose-dependent and saturable manner with an estimated affinity (dissociation constant (K_d) $\approx$ 530 pM; Fig. 2a) identical to that observed for the cloned dopamine D_1 receptor and for human native neuronal membranes²⁴. The rank order of potency for the inhibition of [3 H]SCH-23390 binding to membranes of transfected COS-7 cells by various dopaminergic antagonists and agonists is listed in Table 1 and illustrated in Fig. 2b. Although most dopamine D_1 receptor antagonists and agonists inhibited [3 H]SCH-23390 with a pharmacological profile similar to that observed with the cloned human dopamine D_1 and native membrane-bound receptor, there were notable exceptions. First, dopamine had a 10-fold higher affinity for RS7 than D_1 receptors expressed in COS-7 cells (Table 1). A direct comparison between the affinities of these two receptors for dopaminergic agonists appears valid as neither gene product displays guanine-nucleotide-sensitive agonist high-affinity forms in these cells (data not shown).

FIG. 1 *a*, Restriction map of the human dopamine D₅ receptor genomic clone. Relevant endonuclease restriction sites of the 5-kb RS7 genomic clone. *Xho*I (Xho), *Pst*I (Ps), *Sma*I (S), *Acc*I (A), *Pvu*II (P), *Sac*I (Sac) and *Bgl*II (Bgl), are indicated. Hatched area indicates the coding region of the clone. Putative transmembrane domains are demarcated by Roman numerals above the coding region. *b*, Human dopamine D₅ receptor cDNA. Solid line bars denote polymerase chain reaction (PCR)-generated cDNA fragments from a human putamen λgt11 library which encode the D₅ receptor. *c*, Nucleotide sequence of the coding region of the human dopamine D₅ receptor genomic clone. Nucleotides are presented in the 5' to 3' orientation and the coding region is numbered starting from the putative initiating methionine and ending in the termination codon. Putative transmembrane domains are demarcated by stippled regions and are indicated as TM1-TM7. Putative N-linked glycosylation sites are indicated by triangles. Potential phosphorylation sites for protein kinase A (cAMP-dependent) and protein kinase C are indicated by closed and open squares, respectively. Cysteine 375, the putative site of palmitoylation, is boxed. *d*, Alignment of dopamine receptor primary structure. Boxed regions indicate conserved amino acids between the human dopamine D₅ receptor and other members of the dopamine receptor family. Single-letter amino-acid code used.

METHODS. A 500-bp radiolabelled probe corresponding to transmembrane domains two to five of the human dopamine D₁ receptor was used to screen a human genomic λEMBL3-SP6/T7 library (Clontech). 5 × 10⁵ independent clones were screened under the following hybridization conditions: duplicate nylon filters (Hybond, Amersham) were hybridized at 42 °C in a solution containing ³²P-labelled *Bgl*II-*Hind*III D₁ fragment (2 × 10⁵ c.p.m. ml⁻¹), 0.1% glycine, 3 × SSC buffer, 100 mM sodium phosphate, pH 6.8, 50% deionized formamide, 1 × Denhardt's solution, 10% dextran sulphate and 150 μg ml⁻¹ denatured and sheared salmon sperm DNA (Sigma). Filters were washed under low-stringency conditions at 20 °C for 1 h with 2 × SSC and 0.5% SDS followed by a 1-hour wash at 51 °C. Two identical hybridizing clones of ~22 kb in length were identified and purified (RS5 and RS6). Southern blot analysis of the two clones digested with *Xho*I and hybridized under the conditions described above identified a 5-kb fragment that hybridized to the D₁ fragment. Dideoxynucleotide sequencing (Sequenase V2.0) of the 5-kb *Xho*I fragment subcloned in pSP73 (Promega) revealed an open reading frame containing a putative initiating methionine followed by a stretch of amino acids which are conserved among the G-coupled receptors. The 5-kb fragment contained the entire coding region for the D₅ receptor. Synthetic oligonucleotide primers (Biotechnology Service Center, Toronto) based on the D₅ receptor gene were used to confirm sequence in both directions. To ascertain the possible



existence of introns within the gene, various oligonucleotide primers coding for the D₅ gene were used to amplify cDNA from a human putamen λgt11 library (Clontech) using the PCR under conditions previously described³⁶. Fragments were isolated, subcloned into pSP73 and directly sequenced using vector-driven (SP6-T7) and sequence-specific primers as described above.



Second, (+)butaclamol inhibited [3 H]SCH-23390 binding to the pCD-RS7 gene product with a 10-fold lower affinity (30 nM) than to D₁. The distinct pharmacology of the expressed gene product indicates that this clone encodes a novel dopamine receptor, which we have called D₅.

Whereas all the available data point to the existence of dopamine D₁-like receptors that are not coupled to the stimulation of adenylyl cyclase, the strong overall similarity between

dopamine D₅ and D₁ receptors suggests that this protein, like D₁, functionally couples to G_s. To demonstrate that pCD-RS7 expresses a functional dopamine D₅ receptor, we assessed the ability of dopamine to alter adenylyl cyclase activity in a rat somatomammotrophic GH4-C1 cell line²⁵ transiently expressing pCD-RS7. Dopamine and SKF-82526 (fenoldopam) stimulated adenylyl cyclase in a concentration-dependent manner (Fig. 2c). This stimulation was unaffected by the addition of the specific

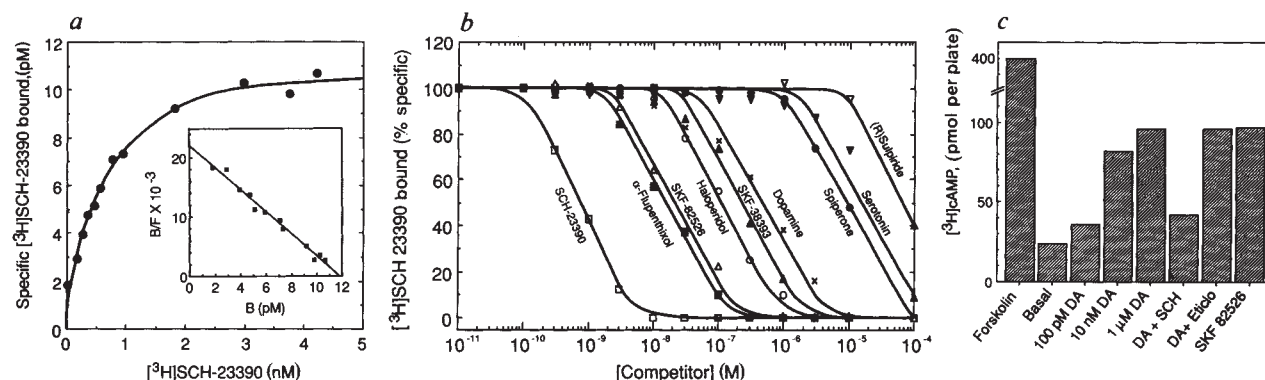


FIG. 2 **a**, Saturation isotherm of [3 H]SCH-23390 binding to membranes prepared from COS-7 cells transformed with pCD-RS7. The results shown are representative of independent experiments each conducted in duplicate. Inset, Scatchard plot of the same data. The estimated maximal binding $B_{\max}$ (receptor density; 412 ± 36 fmol per mg protein), and dissociation constant K_d (550 ± 75 pM) values were obtained by LIGAND as described⁶. **b**, Pharmacological specificity of [3 H]SCH-23390 binding. Representative curves are illustrated for the concentration-dependent inhibition of [3 H]SCH-23390 binding (~ 600 pM) with various dopaminergic agonists and antagonists. Estimated inhibitory constants, K_i , for each drug were determined by LIGAND and the results shown are means of duplicate determinations and are representative of two independent experiments. **c**, Adenylyl cyclase activity of GH4-C1 cells transfected with pCD-RS7. The rat somatomammotrophic cell line GH4-C1 (ref. 25) transiently expressing pCD-RS7 reveals concentration-dependent dopamine stimulation of adenylyl cyclase. Maximal stimulation with 1 μ M dopamine displayed reversibility with the specific dopamine D₁ antagonist SCH-23390 (500 nM) but not with eticlopride (500 nM), a dopamine D₂ receptor antagonist, at an identical concentration. The specific dopamine D₁ agonist SKF-82526 (1 μ M) also stimulated adenylyl cyclase.

Control adenylyl cyclase stimulation was measured by the addition of 10 μ M forskolin. Values are the mean of two determinations with $<18\%$ variation. METHODS. The 5-kb *XhoI* fragment containing the entire coding region of the human dopamine D₅ receptor was subcloned into the mammalian expression vector pCD-PS (ref. 23), yielding pCD-RS7. Caesium chloride-purified pCD-RS7 was introduced into COS-7 cells by calcium phosphate-mediated transfection as described³⁷. Cells were collected and membranes prepared for binding experiments with the dopamine D₁ antagonist [3 H]SCH-23390 as described⁶. For adenylyl cyclase experiments, GH4-C1 cells²⁴ grown in F-10 medium were transfected with pCD-RS7 as described by Chen and Okayama³⁷. Twenty-four hours after transfection, cells were supplemented in fresh media and allowed to grow for 60 h. Collected cells were homogenized and washed in a buffer containing 25 mM Tris-acetate (pH 7.6 at 20 $^{\circ}$ C) and 5 mM magnesium acetate. The ability of various dopaminergic agonists to stimulate adenylyl cyclase was assessed and cAMP accumulation determined as described³⁸. The inhibition of dopamine (1 μ M) stimulated adenylyl cyclase with the selective dopamine D₁ antagonist SCH-23390 (500 nM) and D₂ antagonist eticlopride (500 nM) and stimulation with SKF-82526 (1 μ M) is shown.

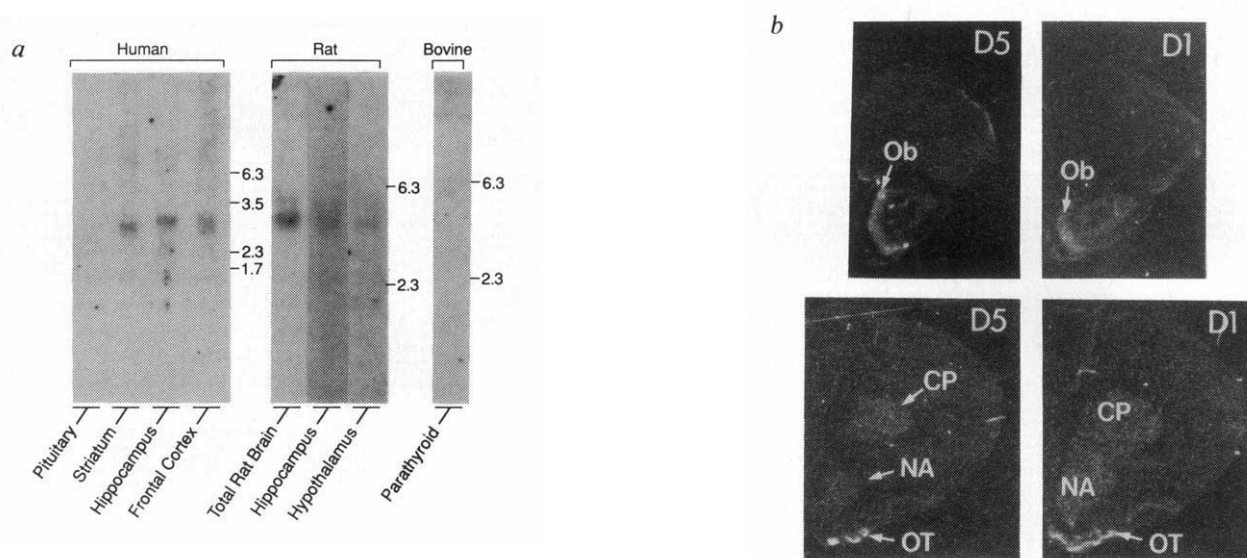


FIG. 3 *a*, Northern blot analysis of the tissue distribution of the dopamine D₅ receptor mRNA. Each lane contains 10 µg polyadenylated RNA from various regions of human and rat brain, as well as the bovine parathyroid gland. Size markers are shown on right in kb. *b*, Comparison of the dopamine D₅ and D₁ receptor mRNA in rat forebrain and midbrain by *in situ* hybridization. Regions that express dopamine D₅ receptor mRNA, including the caudate-putamen, nucleus accumbens, olfactory tubercle and olfactory bulb appear as white in the photomicrograph after a 7-day exposure. Caudate-putamen (CP), nucleus accumbens (NA), olfactory tubercle (OT) and olfactory bulb (Ob) are indicated.

METHODS. *a*, Various regions of human brain, male Wistar rat brains and bovine parathyroid gland were dissected and RNA isolated essentially as described⁶ with the following exception: polyadenylated mRNA was isolated using the mRNA purification kit (Pharmacia) according to the manufacturer's specifications. Poly(A)⁺ RNA was denatured and electrophoresed with glyoxal³⁹, for human RNA, or 6% formaldehyde, for rat and bovine RNA, in a 1% agarose gel and transferred onto nylon membranes (Hybond, Amersham). The blot was hybridized with a 1.2-kb *SmaI-SacI* restriction endonuclease fragment of the D₅ receptor gene which was ³²P-radiolabelled by nick translation (Amersham). Hybridization conditions were as described⁶. The membrane was washed at 20 °C for 1 h with 2 × SSC and 0.1% SDS,

followed by a 60 °C wash for 1 h with 0.2 × SSC and 0.2% SDS. The membrane was finally washed in 0.1 × SSC and 0.1% SDS at 65 °C for 1 h. Blots were exposed to Kodak XAR autoradiographic film with one intensifying screen at -70 °C for 5 to 10 days. *b*, *In situ* hybridization histochemistry was performed essentially as described⁴⁰. Frozen, fixed, 12-µm-thick sections of rat brain were prehybridized for 2 h at 20 °C and then hybridized at 38 °C for 24 h³⁷. Sections were then washed with 2 × SSC for 10 min followed by 2 min in 95% ethanol and air-dried. Radiolabelled human dopamine D₅ receptor probes were prepared from antisense oligonucleotides designed from the RS7 sequence (Biotechnology Services, Toronto). A mixture of three oligonucleotides corresponding to nucleotides 132–156, 282–300 and 1,109–1,133 ((5'-AGATGATGAGTAGGGTCAGCAGGC-3'), (5'-GACTGCCTTCC-AGGGCAT-3') and (5'-AAGTGGCTGCACCCAGCAGCTGG-3'), respectively) were 3' end-labelled with [³⁵S]ATP (NEN) using terminal transferase (BRL). Dopamine D₁ receptor probes were prepared as previously described⁶. Hybridization buffer (50 µl) containing 5 × 10⁵ c.p.m. of labelled probes was applied to each section. After hybridization, slices were rinsed in 0.1 × SSC at 38 °C with four buffer exchanges (4 h) followed by 1 × SSC for 2 h at 22 °C. Sections were dehydrated, dried, and exposed to autoradiographic Dupont MRF-34 Clear film for 7–10 days. As controls, adjacent sections were hybridized with excess unlabelled oligonucleotides or treated with RNase.

D₂ receptor antagonist eticlopride (500 nM), but was completely inhibited by SCH-23390 (500 nM). Adenyl cyclase activity in a nontransfected GH4C1 cell line is not responsive to the presence of dopamine²⁵.

Northern blot analysis of brain and peripheral regions from rat and human polyadenylated mRNA, using a 1.2-kb *SmaI-SacI* fragment of RS6, revealed mRNA transcripts only in brain, particularly within limbic structures (Fig. 3*a*). Unlike dopamine D₁ receptor mRNA, which is expressed in high levels in the basal ganglia, the D₅ transcript is quite rare (about one order of magnitude lower than D₁, as indexed by scanning densitometry); it is equally well expressed in regions such as the frontal cortex, striatum, hippocampus and hypothalamus, and corresponds to the mRNA distribution of DARPP-32, a phosphoprotein regulated by dopamine and cyclic AMP (ref. 26). A similar distribution of D₅ polyadenylated RNA was detected in monkey brain (data not shown). The differently sized D₅ receptor transcripts (evident in Fig. 3*a*) isolated from different regions of both human and rat brain tissues may result from the use of alternative transcription start sites²⁷. D₅ mRNA could not be detected in tissues from the rat pituitary, liver, heart, kidney, lung, spleen, nor in bovine parathyroid gland, the archetype tissue for the dopamine D₁ receptor^{1,3}. Dopamine D₅ and D₁ receptor mRNA was detected in various rat brain

slices by *in situ* hybridization (Fig. 3*b*) using antisense oligonucleotide primers corresponding to the coding sequence. There was some overlap in the distribution of the receptors, with the strongest signals observed in the olfactory tubercle, olfactory bulb, caudate-putamen and nucleus accumbens. The distribution of D₅ receptor mRNA correlates well with traditional dopaminergic pathways²⁸ and the reported distribution of [³H]SCH-23390 binding sites in both rat and human brain^{29–32}.

From our results, we conclude that RS7 encodes a novel dopamine receptor which stimulates adenylate cyclase and has a pharmacological characteristic similar but not identical to the dopamine D₁ receptor. Unlike the D₁ receptor, the higher affinity of the D₅ receptor for the endogenous neurotransmitter dopamine would suggest that this system is responsible for maintaining dopaminergic tone and arousal³³. Interestingly, photoaffinity labelling and peptide mapping experiments have identified a neuronal-specific polypeptide with an *M_r* of about 52,000 which has enhanced affinity for dopamine^{34,35}. The cloning of this receptor will provide a new tool to examine the regulation of dopamine receptors and the functional interaction between D₁/D₅ and D₂/D₃/D₄ receptors. The absence of a restriction fragment length polymorphism using the *XhoI* 5-kb fragment (J. Kennedy, personal communication). 24 enzymes, using 12 unrelated and racially diverse individuals per enzyme)

suggests that the D₅ receptor gene is highly conserved within the human species and may contribute to the maintenance or expression of neuropsychiatric diseases. □

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Reconstitution by MHC-restricted peptides of HLA-A2 heavy chain with β 2-microglobulin, *in vitro*

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CYTOTOXIC T lymphocytes kill virally infected cells when they detect antigenic fragments presented by class I major histocompatibility complex (MHC) antigens (HLA in humans). The crystal structures of HLA-A2 and HLA-Aw68 reveal that peptide-antigen forms an integral part of the HLA structure, being retained in a prominent groove even after purification and crystallization^{1–3}. Here we report that the heavy chain and β 2-microglobulin of HLA-A2, after separation and fractionation in denaturants, reassemble efficiently under renaturing conditions only in the presence of MHC-restricted⁴ peptides. A complex of heavy chain, β 2-microglobulin, and viral peptide in the ratio 1:1:1 is formed in up to 46% yield. Reconstitution is not stimulated by either of two peptides not restricted to HLA-A2. The reconstituted complex of HLA-A2 and the influenza virus (B/Lee/40) nucleoprotein peptide, Np (85–94), crystallizes under conditions previously used to crystallize HLA-A2 (ref. 5). Peptide-linked folding and assembly suggests mechanisms for the unusual capacity of HLA to bind many peptides of diverse sequence.

HLA-A2 molecules were dialysed into denaturant to remove bound and unbound low relative molecular mass components and then the heavy chain and β 2-microglobulin (β 2m) were separated by gel filtration (Fig. 1a and b). To study reconstitution of the HLA-A2 molecules, the separated heavy chain and β 2m were mixed in 6 M sodium thiocyanate with peptides, and the mixture dialysed to remove the denaturant and then analysed by HPLC gel filtration (Fig. 1c and d). SDS-polyacrylamide gel electrophoresis of the fractions indicates a large peak of reconstituted HLA clearly separated from free β 2m and free peptide;

TABLE 1 Sequence of reconstituted A2

Cycle	Yield per cycle (pmol)				Np (85-94)
	Heavy chain		β 2m		
1	G	250	I	300	K 235
2	S	120	Q	137	L 259
3	H	52	R	67	G 208
4	S	149	T	245	E 144
5	M	219	P	139	F 236
6	R	55	K	172	Y 256
7	Y	145	I	152	N 123
8	F	172	Q	(283)*	Q (283)*
9	F	153	V	151	M 154
10	T	160	Y	117	M ND
11	S	196			
12	V	111	R	62	

Amino-acid sequence analysis of gel-filtered HLA-A2 after reconstitution with the Np (85–94). Peak, leading and trailing edge fractions were sequenced. Results for the peak fraction are shown (in single-letter code). Leading and trailing edge fractions contained no discernable sequence and levels of amino-acid yield well below those found in the peak (<20 pmol). After dialysis of 1.1 mg HLA into 6 M NaSCN/MBS (25 mM MES buffer, pH 6.5, 150 mM NaCl) (using a 8000 MWCO membrane for 1.5 h at 25 °C), the heavy chain and β 2m were separated by SW300 gel filtration on HPLC. The separated chains were pooled with peptide (final concentrations: 200 μ M peptide, 6.7 μ M HLA). After dialysis into MBS (using a 500 MWCO membrane for 15 h at 4 °C) and concentration by vacuum dialysis, reconstituted HLA-A2 was purified from unbound peptide by gel filtration in 100 mM ammonium bicarbonate, pH 7.5. Lyophilized peak, leading and trailing fractions were sequenced by Edman degradation.

* Yield of Gln represents sum of β 2m and Np (85–94) sequence at cycle 8.

the free heavy chain aggregates in the absence of denaturants and does not enter the column. With MHC-restricted peptides (influenza nucleoprotein peptide⁶ Np (85–94), P. Robbins, personal communication) or influenza matrix protein peptide⁷ Mp (58–68) the yields on reconstitution were above 40%. In the absence of peptide (Fig. 1e, j and m) (similar to the study by Elliot and Eisen⁸), or with peptides not restricted to HLA-A2, only 5–15% reconstitution was observed. The Np (85–94) and Mp (58–68) peptides did not stimulate reconstitution of class I molecules not known to interact with them in assays based on recognition by cytotoxic T lymphocytes (CTL) (HLA-Aw68, Fig. 1h and i; HLA-B40; Fig. 1k and l; and HLA-B7, data not shown). The results suggest that only some combinations of

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peptides and histocompatibility antigens can reconstitute HLA molecules *in vitro*, that peptide enhances reconstitution under these conditions, and that peptide may be bound to the reconstituted MHC molecules.

The association of peptides with the reconstituted HLA-A2 was determined using radiolabelled Np (85-94) and shown to be specific for peptide sequence. Np (85-94), labelled by [14 C]lysine at its N-terminal position, elutes on gel filtration with reconstituted HLA-A2 (Fig. 2). Unlabelled Np (85-94) and Mp (58-68) could compete with the 14 C-labelled Np (85-94) peptide (Fig. 2) but two unrelated peptides could not, arguing that the Np peptide is bound specifically to HLA-A2. The stoichiometry of peptide: HLA was calculated to be $0.8 \pm 0.1:1$ from the yield of reconstituted HLA-A2, determined by the absorbance at 280 nm, and the total associated radioactivity. Labelled Np peptide incubated under non-denaturing conditions with intact HLA-A2 (in the absence of reconstitution) shows no binding above 1.0% (similar to the results of Chen and Parham⁹).

Radioactive nucleoprotein peptide did not associate with HLA-Aw68, HLA-B40, or HLA-B7 under reconstitution conditions (data not shown), a result consistent with the expectation that peptide binding should demonstrate some specificity with respect to HLA allelic product. The three-dimensional structure of HLA-Aw68 shows that 11 of the 13 amino-acid differences between HLA-Aw68 and HLA-A2 are within the antigen binding groove^{2,3}. The failure of Np (85-94) to stimulate reconstitution of HLA-Aw68, therefore suggests that the Np binding observed here is in the HLA-A2 antigen-binding site.

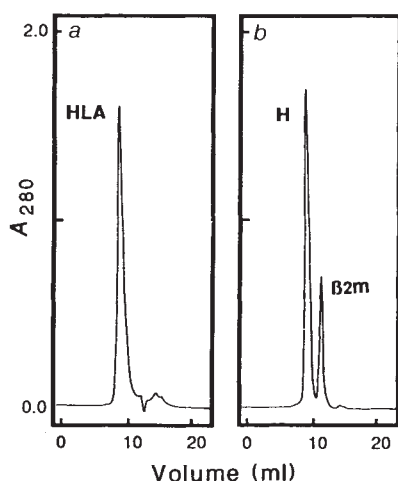
The presence of peptide stoichiometrically bound to reconstituted HLA was also shown by amino-acid sequencing (Table 1). In 10 cycles of Edman degradation, the identity of the amino acids in each cycle were those predicted from the sequences of the HLA-A2 heavy chain, β 2m and Np (85-94). The

yields of amino acids per cycle are consistent with a 1:1:1 complex.

Independent support for the reconstitution of HLA-A2 is provided by isoelectric focusing under native conditions. HLA-A2 reconstituted in the presence of the Np (85-94) or Mp (58-68) peptides are distinguishable from each other on native isoelectric focusing (Fig. 3a, lanes 5 and 6) and from untreated HLA-A2 (lane 1) or HLA-A2 incubated with these peptides in non-reconstitution conditions (lanes 2 and 3), presumably reflecting the isoelectric point of peptide-HLA complexes present in each case. (The multiple bands observed with reconstituted HLA are due to heterogeneous sialylation, as these can be reduced to one main band after neuraminidase treatment (data not shown; see legend to Fig. 3).) The band-shifting characteristic of reconstitution was not observed in the absence of peptide (lane 4) or in the presence of two non-HLA-A2-restricted peptides (lanes 7 and 8). HLA-B7 (lane 9) did not reconstitute in the absence of peptide (lane 10) or in the presence of the HLA-A2-restricted peptides Np (85-94) and Mp (58-68) (lanes 11 and 12). The native isoelectric focusing assay thus also indicates that the reconstitution is both allele- and peptide-specific.

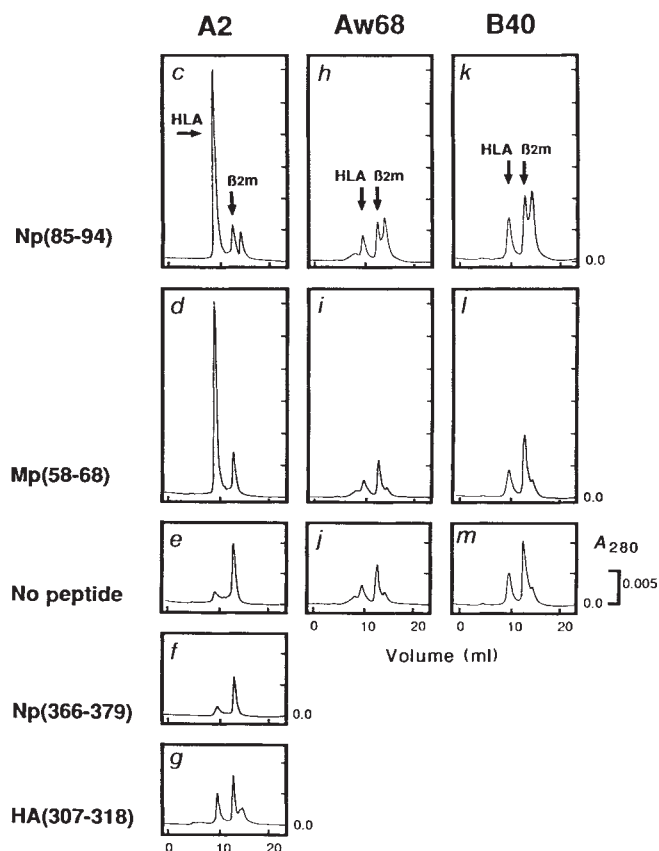
Immunoblotting and autoradiography of the native isoelectric focusing gels demonstrates that the shifted bands are formed by complexes containing the HLA-A2 heavy chain, light chain and radiolabelled Np (85-94). An immunoblot (Fig. 3b) with antibodies to heavy chain (left panel) and to β 2m (right panel), shows that both subunits are present in the molecular complexes reconstituted with Np (lane 2) and Mp (lane 3) peptides. The autoradiogram shown in Fig. 3c demonstrates both the presence of radiolabelled Np (85-94) in reconstituted HLA-A2 (lane 3) and the specificity of the reconstitution with respect to peptide (lanes 4-7) and histocompatibility allele (lanes 8 and 9). Binding of radioactive Np (85-94) to HLA-A2 in the absence of reconstitution (lane 10) is barely detectable.

FIG. 1 Gel filtration of HLA-complex reconstitution. *a*, Untreated HLA-A2. *b*, HLA-A2 dialysed in NaSCN/MBS. *c*, H, heavy chain. *c*-*g*, NaSCN-treated HLA-A2. *h*-*j*, NaSCN-treated HLA-Aw68. *k*-*m*, NaSCN-treated HLA-B40. Dialysis performed in MBS with Np (85-94) (*c*, *h* and *k*), Mp (58-68) (*d*, *i* and *l*), no peptide (*e*, *j* and *m*), control peptides Np (366-379) (*f*) and HA (307-318) (*g*). Peak compositions determined by SDS-PAGE; free heavy chain is



insoluble and does not enter the column in the absence of denaturants. Reconstitution yields, triplicate runs: $46 \pm 6\%$ A2-Np (85-94); $40 \pm 6\%$ A2-Mp (58-68), 5-15% for non-restricted pairs, or no peptide. In the single-letter amino-acid code, Np(85-94) is KLGEFYNQMM; Mp(58-68), GILGFVFTLT, Np(366-379), ASNENMETMESSTL; HA(307-318), PKYVKQNTLKLAT.

METHODS. After dialysis of 1.1 mg HLA, papain-solubilized as described²⁰, into 6 M NaSCN, 25 mM MES, pH 6.5 (with NH_4OH), 150 mM NaCl (NaSCN/MBS) (using a 8000 MWCO membrane for 1.5 h at 25 °C), heavy chain and β 2m were separated by HPLC gel filtration in NaSCN/MBS (Waters Protein Pak SW300). Chains were pooled in a 1:1 molar ratio and added to peptides (final: 200 μM peptide, 6.7 μM HLA). Np (85-94) and Mp (58-68) are restricted to HLA-A2. Np (366-379) is restricted to mouse H2-D^b (refs 15, 21), HA (307-318) to human class II DR1 (ref. 22). After dialysis into MBS (using a 500 MWCO membrane for 15 h at 4 °C), samples were analysed by gel filtration in MBS. Reconstitution yield is calculated as the integrated peak of the absorbance at 280 nm (A_{280}) for the reconstituted complex/integrated A_{280} peak for starting HLA. Peptides were synthesized using 0.25 mmol-



scale (N-(9-fluorenyl-methoxycarbonyl; fmoc) chemistry (ABI Model 431A) and purified by reverse-phase HPLC.

A clear indication that reconstituted HLA-A2 plus Np (85-94) peptide has the same structure as the native HLA-A2 is that it crystallizes under the same conditions as native HLA-A2 into morphologically similar plate-crystals⁵. Native isoelectric focusing of protein from solubilized crystals exhibits the shifted set of bands (as in Fig. 3a, lane 5), indicative of the presence of peptide Np (85-94). Reconstituted HLA-A2 complexes were also recognized by the conformation-sensitive antibody PA2.1 (ref. 10) enzyme-linked immunosorbent assay, a further indication that the complexes are in a native conformation (data not shown).

In contrast to a measurement of direct peptide to HLA binding in solution⁹, which demonstrated binding of less than 0.3% of HLA molecules, *in vitro* reconstitution after denaturation shows over 40% reconstitution of HLA stoichiometrically complexed with peptide. This increase in binding efficiency is presumably due to the removal of endogenous peptides and a requirement for simultaneous binding and refolding. Unlike a solid-phase binding assay¹¹⁻¹³, the *in vitro* reconstitution shows allelic specificity in peptide binding as expected from CTL recognition experiments (see, for example, ref. 14).

A central structural puzzle in histocompatibility-restricted antigen recognition⁴ is the nature of the mechanism by which one histocompatibility glycoprotein can form long-lived complexes with many different peptide antigens. The linkage

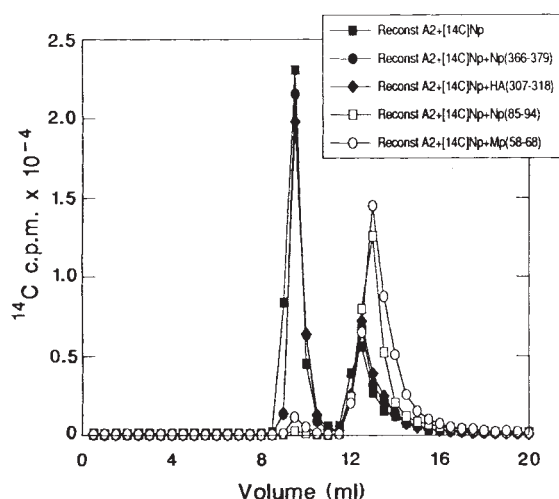


FIG. 2 Gel filtration of HLA reconstituted with ^{14}C -labelled Np (85-94) and competition of unlabelled peptides for association with HLA-A2. Reconstitution of HLA-A2 was carried out in the presence of ^{14}C -labelled Np (85-94) or ^{14}C -labelled Np (85-94) with excess unlabelled Np (85-94), Mp (58-68), Np (366-379) or HA (307-318).

METHODS. Reconstitution was as described for Fig. 1 using 100,000 c.p.m. ^{14}C -labelled Np (85-94) and unlabelled peptide as indicated (final concentrations: 1.5 μM ^{14}C -labelled Np (85-94), 6.7 μM HLA, 200 μM unlabelled peptide). After dialysis, samples were analysed by gel filtration in MBS (as for Fig. 1). Radioactivity was measured by liquid scintillation counting. HLA was monitored by absorbance at 280 nm. Synthesis of ^{14}C -labelled Np (85-94): Di-fmoc- ^{14}C lysine was synthesized from ^{14}C lysine (305 Ci mol^{-1} ; NEN) (ref. 23: as fmoc-L-tyrosine procedure, 1:1,000 scale, filtration and recrystallization replaced with ethyl acetate extraction). Di-fmoc- ^{14}C lysine was confirmed by TLC with non-radioactive di-fmoc-lysine (Bachem). (CH_2Cl_2 :methanol:acetic acid, 90:8:2, R_f 0.50). Di-fmoc- ^{14}C lysine was activated by adding 265 μl 3.8 mM dicyclohexylcarbodiimide (DCC), 1-hydroxybenzotriazole (HOBt) and pentafluorophenol in *N*-methylpyrrolidone (NMP) with occasional mixing (30 min, 25 $^\circ\text{C}$). We added 15 mg fmoc-deprotected Np (86-94) resin in 160 μl NMP and stirred overnight at 25 $^\circ\text{C}$. The resin was washed with NMP, deprotected and cleaved (ABI 431A manual 100-mg scale). Ratio of peptide bound to reconstituted HLA-A2 was [(total μCi in HLA peak)/(specific activity of ^{14}C Np (85-94))]:[(integrated A_{280} of HLA peak)/(extinction coefficient HLA-A2)]. Specific activity of ^{14}C -labelled Np (85-94) is 304 μCi μmol^{-1} . Extinction coefficient of HLA-A2 is 92,250 $\text{cm}^{-1} \text{M}^{-1}$ at 280 nm.

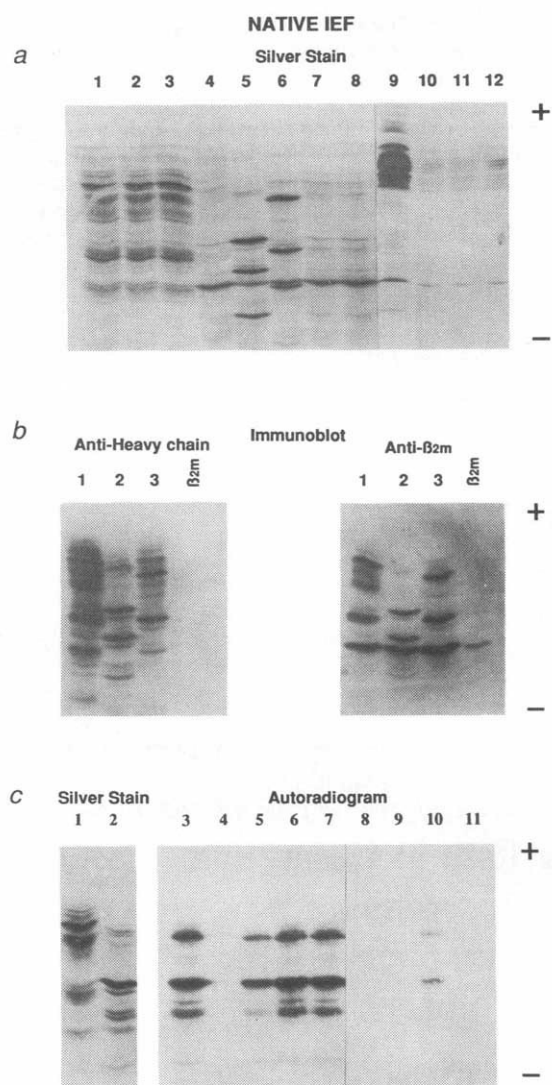


FIG. 3 Native isoelectric focusing (IEF) a, Silver-stained native IEF: HLA-A2 (lane 1); HLA-A2 incubated in non-reconstituting conditions with 200 μM Np (85-94) (lane 2) or Mp (58-68) (lane 3). 'Reconstituted' HLA-A2: no peptide (lane 4), Np (85-94) (lane 5); Mp (58-68) (lane 6), and non-HLA-A2-restricted peptides Np (366-379) (lane 7) and HA (307-318) (lane 8). HLA-B7 (lane 9). 'Reconstituted' HLA-B7: no peptide (lane 10), Np (85-94) (lane 11), Mp (58-68) (lane 12). HLA-A2 (lanes 1-3) and dissolved crystals of HLA-A2 are heterogeneous by native IEF. The heterogeneity could be from sialic acid, deamidations and carbamylations of HLA, and/or the presence of multiple peptides. The pattern varies with different preparations of HLA-A2. Reconstituted complexes exhibit a distinct and simpler pattern (as, for example, in lanes 5 and 6), possibly resulting from the substitution of one peptide for a mixture. b, Native IEF immunoblot. Left: anti-heavy-chain blot, HLA-A2 (lane 1), HLA-A2 reconstituted with Np (85-94) (lane 2), Mp (58-68) (lane 3), $\beta_2\text{m}$. Right: anti- $\beta_2\text{m}$ blot with same samples. c, Native IEF autoradiogram. Left: silver stain, native HLA-A2 (lane 1), HLA-A2 reconstituted with Np (85-94) (lane 2). Right: autoradiogram of HLA-A2 reconstituted with ^{14}C -labelled Np (85-94) (lane 3), with ^{14}C -labelled Np (85-94) and 200 μM Np (85-94) (lane 4) with Mp (58-68) (alanine substituted at position 64) (lane 5) with A2 (102-117) (lane 6), or with Np (366-379) (lane 7). HLA-B40 'reconstituted' with ^{14}C -labelled Np (85-94) (lane 8), HLA-Aw68 'reconstituted' with ^{14}C -labelled Np (85-94) (lane 9), HLA-A2 incubated with ^{14}C -labelled Np (95-94) without reconstitution (lane 10) and ^{14}C -labelled Np alone (lane 11). $\beta_2\text{m}$ position ●.

METHODS. Reconstitution was as described for Fig. 1; IEF, was run from pH 5-7. For immunoblotting, we used anti-HLA A,B,C (Olympus)²⁴ and anti- $\beta_2\text{m}$ BM-63 (Sigma) mouse monoclonal antibodies and alkaline phosphatase anti-mouse antibody (Promega). For autoradiography, the IEF gel was dried and exposed to X-omat film at -70 $^\circ\text{C}$ with an intensifying screen. Reconstitution for the autoradiogram included 20,000 c.p.m. ^{14}C -labelled Np (85-94).

between antigenic peptide binding and HLA folding and/or assembly has now been found in living cells¹⁵, cell lysates^{16,17} and here, with purified molecules. This linkage implies that HLA-restricted peptides generate stable HLA heavy-chain/ β 2m complexes either by stabilizing the HLA- β 2m heterodimer or by interacting with the HLA heavy chain and facilitating β 2m binding to form a stable complex, or both. A discussion of specific interactions between the HLA-A2 heavy chain and β 2m and how their folding and assembly might be peptide-dependent

is given by Saper *et al.*¹⁸. Because peptide binding appears to be linked to HLA folding, the sequence diversity of peptides able to bind may be expected to parallel the sequence degeneracy found in similarly folded protein structures¹⁹.

Note added in proof. Peptide-dependent reconstitution of a soluble construct of the HLA-A2 heavy chain and β 2m, purified separately from expression in *Escherichia coli*, has recently been observed (K.C.P., M.L.S. and D.C.W., manuscript submitted). □

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Somatotroph hypoplasia and dwarfism in transgenic mice expressing a non-phosphorylatable CREB mutant

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MOST of the transcriptional effects of cyclic AMP are mediated by the cAMP response element binding protein (CREB)^{1,2}. After activation of cAMP-dependent protein kinase A, the catalytic subunits of this enzyme apparently mediate the phosphorylation and activation of CREB^{3,4}. As cAMP serves as a mitogenic signal for anterior pituitary somatotrophic cells⁵, we investigated whether CREB similarly regulates proliferation of these cells. We prepared transgenic mice expressing a transcriptionally inactive mutant of CREB (CREBM1), which cannot be phosphorylated, in cells of the anterior pituitary. If CREB activity is required for proliferation, the overexpressed mutant protein would effectively compete with wild-type CREB activity and thereby block the response to cAMP. As predicted, the CREBM1 transgenic mice exhibited a dwarf phenotype with atrophied pituitary glands markedly deficient in somatotrophs but not other cell types. We conclude that transcriptional activation of CREB is necessary for the normal development of a highly restricted cell type, and that environmental cues, possibly provided by the hypothalamic growth hormone-releasing factor, are necessary for population of the pituitary by somatotrophic cells.

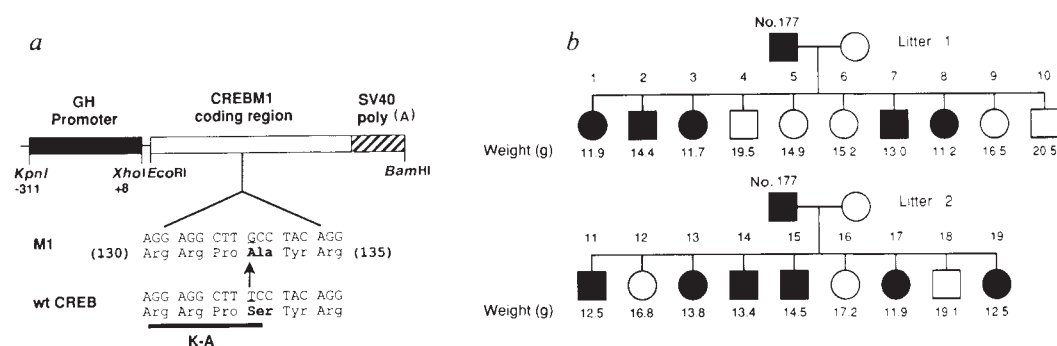
CREB contains a single protein kinase A phosphoacceptor site (Ser 133) which is necessary for transcriptional activation

by cAMP. The CREBM1 cDNA contains a missense mutation which changes Ser 133 to Ala. This mutant gene was inserted into a eukaryotic expression vector containing a 320-base pair (bp) (*KpnI/Xho*; –310/+8) fragment of the rat growth hormone (GH) promoter, which directs expression of transgenes to the somatotrophs of transgenic mice^{6–8}. Following microinjection of the GH-CREBM1 fusion gene (Fig. 1a) we obtained two transgenic animals of reduced size at 8 weeks of age. Although the first of these died prematurely, the second sired two litters totalling 19 F₁ offspring, 11 of which carried the transgene. All animals bearing the GH-CREBM1 transgene exhibited a dwarf phenotype (Fig. 1b) and differences in weights of transgenic and normal littermates were apparent at as early as 3 weeks of age (Fig. 2a). Both sexes were affected: males showed a 31% reduction in body weight at 6 weeks of age (transgenic, 13.6 ± 0.4 g, mean ± s.e.m. for N = 5; control, 19.7 ± 0.4 g, mean ± s.e.m. for N = 3) and females showed a 24% reduction (transgenic, 12.2 ± 0.4 g, mean ± s.e.m. for N = 6; control, 16.1 ± 0.5 g, mean ± s.e.m. for N = 5). The extent of growth retardation was comparable to that of other studies in which somatotroph lineages were ablated using either thymidine kinase⁸ or diphtheria toxin⁶.

Macroscopic examination of transgenic pituitaries showed normal posterior and intermediate lobes, but atrophied anterior lobes (Fig. 2b). Immunohistochemical analysis revealed a large depletion of somatotrophic cells and hypertrophy of the remaining GH-staining cells (Fig. 3). As somatotrophs are normally the predominant cell type in the anterior lobe, their virtual absence from these transgenic mice would seem to account for the observed atrophy. Consistent with this, corticotroph, gonadotroph and thyrotroph cell densities were elevated. Surprisingly, lactotroph cell densities were apparently normal or only slightly reduced and, moreover, represented the dominant cell type populating the anterior lobes of transgenic mice. Thus, in contrast to results obtained using thymidine kinase and diphtheria toxin transgenes, where both somatotroph and lactotroph lineages were either eliminated or severely reduced^{6,8}, the GH-CREBM1 mutant seems preferentially to limit clonal expansion of somatotrophic cells.

To understand the basis of this selectivity, we examined the expression of the CREBM1 protein in transgenic tissues.

FIG. 1 Generation of GH-CREB1 transgenic mice. *a*, Structure of the GH-CREB1 fusion gene used for microinjection. The Ser 133 to Ala mutation is indicated by an arrow with the affected amino acid in bold type. The protein kinase A recognition sequence is indicated by a solid bar. *b*, Pedigree analysis of GH-CREB1 F₁ offspring of founder number 177. Male offspring are indicated by a square and females by a circle. Animals bearing the transgene are shown as filled. Weights at 6 weeks of age are given below each animal.



METHODS. A plasmid containing the GH-CREB1 fusion gene was constructed by isolation of a 320-bp (*KpnI*-*XhoI*) fragment of the GH promoter and a 1.5-kilobase (kb) (*EcoRI*-*BamHI*) fragment containing the CREB1 coding region and SV40 polyadenylation site⁴ followed by sequential ligations into the polylinker region of Bluescript SK(Stratagene). The GH-CREB1 fusion gene for microinjection was prepared by purification of a 1.8-kb (*Bss*HII-*BamHI*) fragment by agarose gel electrophoresis and further purified using

sucrose gradient centrifugation. This fragment was microinjected into the male pronuclei of fertilized mouse eggs, and the injected eggs transplanted to pseudopregnant foster mothers following standard procedures²⁰. Potential founders were screened by dot-blot analysis of tail DNA using a random primer labelled 250-bp fragment containing the SV40 polyadenylation signal as a probe. The small founder, number 177, was mated to two non-transgenic females to provide F₁ offspring for subsequent analysis. All mice were maintained in a pathogen-free environment and provided with food and water *ad libitum*.

FIG. 2 Effect of somatotroph expression of CREB1 transgene on somatic growth and pituitary development. *a*, Weight history of GH-CREB1 transgenic mice (●, *n* = 5) and non-transgenic littermates (○, *n* = 3). Points are mean ± s.e.m. for male F₁ offspring from two simultaneous litters. *c*, Comparison of the pituitary glands of control (top) and GH-CREB1 transgenic mice (bottom). p, Posterior lobe; a, anterior lobe.

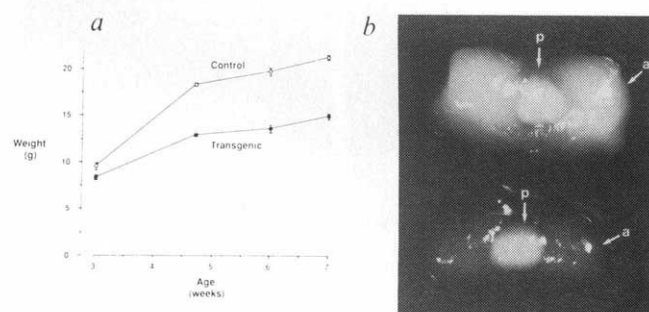
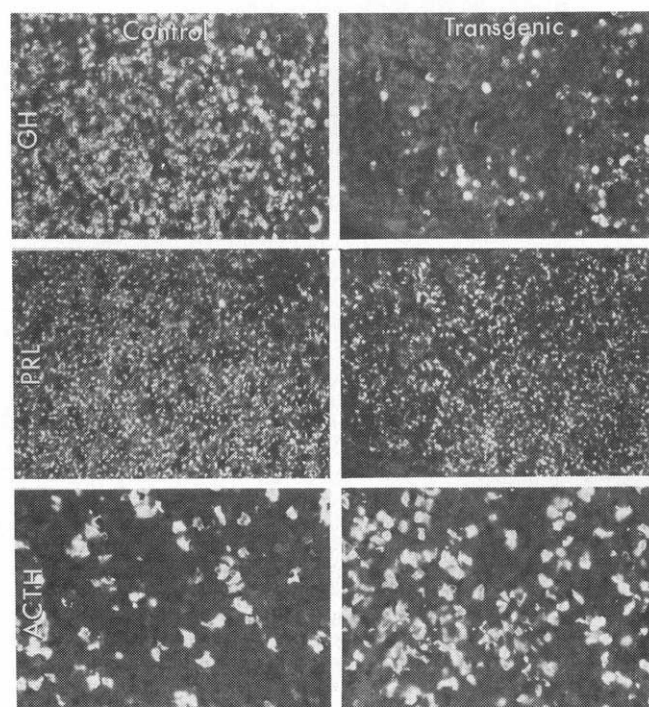


FIG. 3 Expression of pituitary hormones in control and GH-CREB1 transgenic mice. Comparison of indirect immunofluorescence staining patterns for growth hormone (GH), prolactin (PRL) and adrenocorticotrophic hormone (ACTH) in control and GH-CREB1 transgenic mice. A marked decrease in the somatotroph population is evident in transgenic animals; many residual GH-immunoreactive cells are hypertrophied. By contrast, the lactotroph population of the anterior lobes of transgenic is roughly comparable to that of controls, but the density of corticotrophs is increased, presumably as a consequence of the overall reduction in the size of the gland. Magnification for all micrographs, ×150.

METHODS. Mice were anaesthetized with chloral hydrate and perfused with neutral-buffered 10% formalin. Pituitaries were removed and postfixed overnight at 4 °C in the same fixative with 10% sucrose added. Twenty μm-thick frozen sections were stained using a conventional indirect immunofluorescence protocol²¹. Primary antisera included monkey anti-mouse GH (1:80,000 dilution; provided by Y. Sinha, The Whittier Institute), rabbit anti-human prolactin (1:1,000; Cambridge Medical Diagnostics, Billerica, Massachusetts) or rabbit antisera against ACTH1-24, rat thyroid-stimulating hormone (1:2,000), rat luteinizing hormone (1:5,000) and rat follicle-stimulating hormone (1:2,000), all obtained from the National Pituitary and Hormone Distribution program of the NIADDK. Primary antisera were localized using affinity-purified, fluorescein- or rhodamine-conjugated goat anti-mouse (Cappel, Pennsylvania) or anti-rat IgG (Tago, California).



Immunohistochemical staining with CREB-specific antisera showed strong nuclear staining for CREB throughout most of the anterior lobe in transgenic animals but only low levels of diffuse staining in nontransgenic controls (Fig. 4a). By contrast, the intensity of CREB staining in cells of the intermediate lobe was comparable in control and transgenic mice. Western blot analysis of nuclear extracts from transgenic and control pituitaries showed a single CREB immunoreactive protein with a relative molecular mass of 43,000 which was more abundant in the transgenic extract (Fig. 4b). To distinguish between the expression of wild-type (phosphorylatable) and mutant (non-phosphorylatable) CREB in these tissues, we prepared

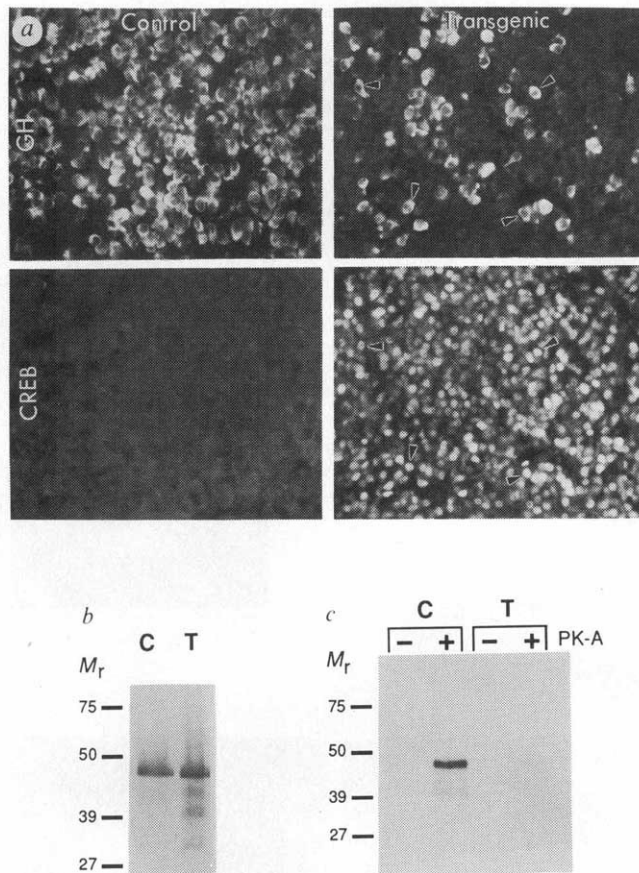


FIG. 4 Expression of mutant CREB in the pituitaries of control and GH-CREB M1 transgenic mice. **a**, Dual immunostaining for GH and CREB in anterior pituitaries of control and transgenic mice. A strong nuclear localization of CREB-immunoreactivity is detected in (but not restricted to) somatotrophs of transgenic but not control mice. Similarly oriented arrowheads in the top and bottom panels mark examples of doubly stained cells. **b**, Western blot analysis of CREB proteins in pituitary nuclear extracts of control (c) and transgenic (t) mice. **c**, Protein-kinase A phosphorylation of immunoprecipitated CREB protein from pituitary nuclear extracts of control (c) and transgenic (t) animals. M_r , relative molecular mass calibration (in thousands). **METHODS**. Sections and immunostaining were performed as described in Fig. 3. Nuclear CREB protein was localized using affinity-purified rabbit CREB-specific antiserum W39 (ref. 2); this was applied to sections concurrently with monkey anti-mouse GH. Nuclear extracts were prepared from pools of whole pituitaries (anterior, intermediate and posterior lobes) from control and transgenic animals. For western blot analysis, protein (20 μ g) was fractionated on a 10% SDS-polyacrylamide gel, transferred to nitrocellulose, labelled with CREB-specific antisera W39, and visualized using second antibody conjugated to horseradish peroxidase. Immunoprecipitation and 32 P-labelling with protein kinase A was carried out as previously described² on the same nuclear extracts (20 μ g).

immunoprecipitates from control and transgenic samples after phosphorylation with catalytic subunit of protein kinase A and [γ - 32 P]ATP. SDS-PAGE of control extracts revealed a prominent band corresponding to the 32 P-labelled 43K CREB protein, but only a faintly detectable band in transgenic extracts (Fig. 4c). Thus, most of the immunoreactive CREB protein in transgenic extracts seems to be the product of the mutant transgene rather than the endogenous murine gene whose product is able to be phosphorylated.

How does expression of the mutant CREB M1 protein prevent the development of the somatotroph lineage in the transgenic mice? The number of anterior pituitary cells expressing the mutant protein far exceeds the number of residual growth-hormone producing cells, indicating that other cell types, most notably the lactotrophs, must also express this mutant. The specific reduction in the somatotroph population, however, would argue against a simple toxic effect arising from overexpression of the mutant CREB protein. Moreover, stable cell lines that express CREB or inactive mutants of CREB such as M1 grow normally⁹ (M.M., unpublished results). As phosphorylation of CREB apparently does not modulate DNA binding activity, the dramatic reduction in somatotroph numbers that we observed when CREB M1 was overexpressed can be attributed to a loss of transcriptional activity accompanying the removal of the protein kinase A phosphoacceptor site at Ser 133. Although the targets of CREB action are not known, expression of the *c-fos* proto-oncogene¹⁰ and *GHF1/pit-1* genes^{11,12} have previously been shown to be responsive to cAMP. The well documented roles of these proteins in regulating proliferation¹³ and pituitary-specific gene expression¹⁴⁻¹⁶, respectively, suggests that repression of these genes in the transgenic mice might contribute to the observed phenotype.

The ability of a mutant CREB gene to block somatotroph proliferation suggests a novel function for this transactivator. It is tempting to speculate that constitutively active mutants of CREB, or other components of the cAMP transduction system, may stimulate uncontrolled expansion of somatotrophs. In this regard, chronic overstimulation of the somatotroph cAMP pathway is sufficient to cause hyperplasia of these cells^{17,18}. Furthermore, several patients with somatotroph tumours express constitutively activated mutant forms of the G_s protein¹⁹ and to have correspondingly elevated levels of cAMP in transformed cells. Our results suggest that the proliferation of these cells may, at least in part, be caused by constitutive phosphorylation and activation of CREB. □

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Processing of the precursor of NF- κ B by the HIV-1 protease during acute infection

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TRANSCRIPTION of the human immunodeficiency virus type-1 (HIV-1) genome is regulated in part by cellular factors and is stimulated by activation of latently infected T cells. T-cell activation also correlates with the induction of the factor NF- κ B which binds to two adjacent sites in the HIV-1 long terminal repeat¹. This factor consists of two DNA-binding subunits of relative molecular mass 50,000 (50K) associated with two 65K subunits. It is located in the nucleus in mature B cells, but is present in other cell types as an inactive cytoplasmic complex^{2,3}. External stimuli, including those that activate T cells, result in nuclear translocation of active NF- κ B. The cloning of the complementary DNA for the 50K subunit^{4,5} helped to identify an exclusively cytoplasmic 105K precursor (p105) (V.B., P.K. and A.I., manuscript submitted). The expression of active NF- κ B might therefore also be regulated by the extent of processing of p105. Because HIV-1 requires active NF- κ B for efficient transcription¹, we tested the effect of HIV-1 infection on the processing of the human 105K precursor. We show here that the HIV-1 protease can process p105 and increases levels of active nuclear NF- κ B complex.

We infected CEM human T cells with HIV-1 and labelled them with [³⁵S]methionine at various times after infection. Cytoplasmic extracts were immunoprecipitated using p50-specific antisera. Three days after infection, the cells contained less 105K precursor than the uninfected control (Fig. 1a, lanes 1 and 3; c, shows the specificity of the antiserum). This decrease is correlated with the appearance of both the physiological p50 maturation product and a protein, p45, migrating slightly faster than normal p50 (lane 3).

To investigate whether the HIV-1 protease is involved in this phenomenon, we treated labelled cytoplasmic extracts from uninfected CEM cells with purified HIV-1 protease and analysed them by immunoprecipitation. Both p105 and p50 are processed and almost completely converted into the p45 form (Fig. 1b, lane 1). This compares with what is found three days after HIV-1 infection, although *in vitro* processing by the HIV-1 protease is more extensive than that observed *in vivo*. We then digested various derivatives of human p105 (obtained by *in vitro* transcription-translation of 3' truncations of the full-length cDNA) with the HIV-1 protease. Whereas p105 is faithfully processed in the lysate, digestion with the protease produced a protein which is slightly smaller than p50 (Fig. 2). The same band can be detected irrespective of the length of the primary product, suggesting that p45 shares its N-terminus with p105 and its C-terminal truncated derivatives, and ends slightly upstream of the physiological processing point.

We then assayed various extracts derived either from infected or from non-infected cells. Nuclear extracts from cells three days after infection (Fig. 3a, lanes 3 and 4) yield a complex migrating slightly faster than the one observed with purified NF- κ B (which comprises two p50 DNA-binding subunits associated with two p65 subunits: lane 6(-)). Uninfected cells (or cells infected for 20 h: lanes 1 and 2) show only a weak band corresponding to NF- κ B. More DNA-binding activity is found in nuclei from cells infected for three days, which corre-

lates with the more extensive processing of p105 shown in Fig. 1a. Lanes 8–10 and 11–13 show an assay for AP1- and NF1-binding activities, respectively, during HIV infection of CEM cells as in lanes 1–3; there was no significant qualitative or quantitative variation in these activities. Purified human NF- κ B (provided by P. Baeuerle) or p50/KBF1 (ref. 4) were then treated with purified protease. Figure 3a (lanes 5–6) shows an increase in mobility of both NF- κ B- and p50-specific complexes after digestion with the protease, which correlates with the appearance of p45 in Figs 1 and 2. We found no difference in the migration rate of mouse p50/KBF1 (ref. 6) (lanes 7 – and +), suggesting that mouse p50 is not cleaved by the HIV-1 protease. This was confirmed by *in vitro* digestion of various products translated *in vitro* from the cDNA coding for the mouse p50 precursor (provided by S. Ghosh and D. Baltimore, data not shown). Finally, *in vitro* experiments performed on crude extracts from COS cells indicate that the monkey p105 precursor and p50 maturation product can also be processed by the HIV-1 protease yielding a product identical to human p45 (data not shown).

To confirm the role of the HIV-1 protease we transfected COS cells with an expression vector for the p105 precursor (V.B., P.K. and A.I., manuscript submitted), followed by infection with a recombinant vaccinia virus encoding the HIV-1 pol precursor. Bandshift assays on nuclear extracts from these infected cells, using wild-type vaccinia virus-infected cells as a control (Fig. 3b, lanes 1 and 2), showed increased mobility of the NF- κ B complex similar to that observed after HIV-1 infection of CEM

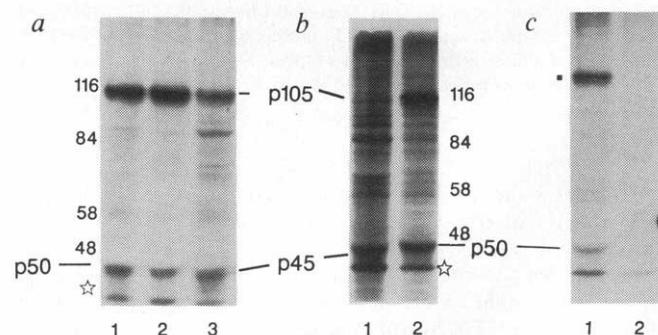


FIG. 1 Immunoprecipitation of cytoplasmic extracts derived from CEM cells infected with HIV-1 and labelled at various times after infection. Lane 1, non-infected cells; lane 2, infected cells 20 h after infection; lane 3, infected cells 68 h after infection. The positions of the p105 precursor, p50 and p45 are indicated by arrows. *M_r* markers are indicated (in thousands). The p50 protein migrates slightly faster than the 48K marker in this system, but the use of other sets of markers or other acrylamide concentrations, in correlation with the accurate calculation of the size of the HIV-1 protease digestion product, confirm a molecular weight of 46–47K for p50. The star indicates a nonspecific complex precipitated by the antiserum. *b*, Immunoprecipitation of cytoplasmic extracts derived from uninfected cells, and treated *in vitro* with purified HIV-1 protease. Lane 1, extract treated with the protease; lane 2, untreated extract. The film has been overexposed to reveal the residual amount of p105 in lane 1. *c*, Specificity of the antiserum. Labelled cytoplasmic extracts were immunoprecipitated with antiserum number 3 (ref. 4) in the absence (lane 1) or presence (lane 2) of 4 μ g of bacterially expressed fusion protein containing amino acids 1–400 of p105. The filled square indicates p105.

METHODS. CEM cells (40×10^6) were infected with a CEM-adapted derivative of HIV-1 Bru¹¹. At 4 h (time 20 h) or 52 h (time 68 h) after infection the medium was replaced with RPMI without methionine, supplemented with 2% dialysed fetal calf serum. After 30 min, 100 μ Ci per ml [³⁵S]methionine were added for 16 h. Control cells were labelled similarly. After labelling, cytoplasmic and nuclear fractions were prepared. About 100 μ g cytoplasmic fraction were boiled for 5 min in 1% SDS, immunoprecipitated with antiserum number 3 (ref. 4) and the resulting products separated by 12% SDS-PAGE. For *in vitro* HIV-1 protease treatment, 100 μ g of cytoplasmic extract derived from uninfected cells were treated with 200 ng of purified protease¹² in 0.1 M acetate buffer, pH 5, for 1 h at 37 °C, followed by immunoprecipitation as described.

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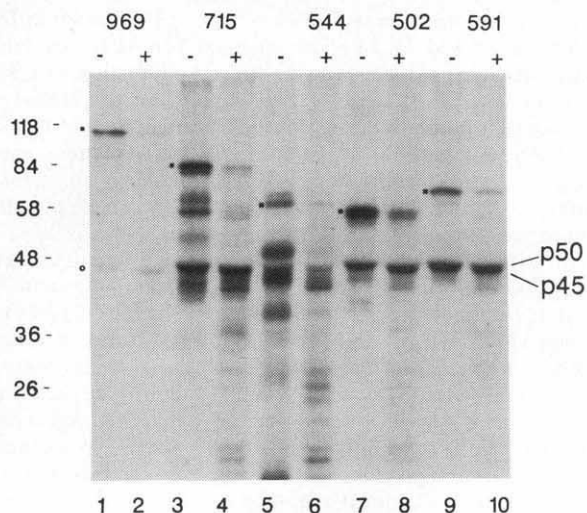


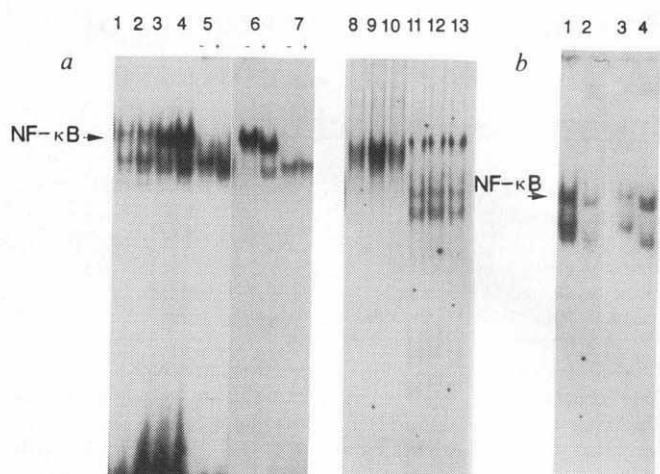
FIG. 2 Digestion with HIV-1 protease of various derivatives of the p50 precursor truncated at the C terminus. The length of each derivative (in amino-acid residues) is indicated above each pair of lanes. +, Protease-treated samples, and -, mock-treated samples. The band corresponding to the primary translation product is indicated by a filled square, the p50/p45 area is indicated by an open circle.

METHODS. Various C-terminal truncations of the cDNA coding for the full-length 105K precursor protein were transcribed and translated in rabbit reticulocyte lysate as described in ref. 4. Each translation product (2 μ l) was incubated for 1 h at 37 °C in 20 μ l of 0.1 M acetate buffer, pH 5, with or without 60 ng of purified HIV-1 protease. This was followed by immunoprecipitation with antiserum number 3 (ref. 4) and analysis by SDS-PAGE as described in the legend to Fig. 1.

cells (lanes 3 and 4). The reason for the high amount of nuclear NF- κ B after infection by wild-type vaccinia virus (lane 1) is not known.

To localize the site of cleavage by the HIV-1 protease, we cloned a fragment of the p105 precursor (amino acids 341 to 502) into the pGEX vector, purified the recombinant protein from *Escherichia coli* as described⁴ and digested it *in vitro* with the viral protease. The C-terminal fragment was isolated and its N terminus sequenced to yield the sequence Pro-Thr-Tyr, localizing the cleavage site to the C-terminal side of Phe 412 (ref. 4). This is consistent with previous findings that the HIV-1 protease often cleaves between Phe and Pro^{7,8}. The mouse p105, which is not cleaved by the viral protease *in vitro*, has a divergent sequence in this region.

Our results suggest that during HIV-1 infection the viral protease processes the 105K precursor of p50, as well as p50 itself, into a p45 maturation product corresponding to amino acids 1 to 412. The p45 protein still binds DNA and interacts with p65 (Fig. 3a, lanes 6 - and +), indicating that the NF- κ B complex observed during infection is probably functional. Although the p45 subunit may remain cytoplasmic through interaction with p65 and I κ B (ref. 2), this would still ensure a larger pool of rapidly available NF- κ B which can be quickly translocated into the nucleus following an external stimulus^{9,10}. The results shown in Fig. 3a (lanes 1-4) indicate that some of the newly processed p45 escapes cytoplasmic retention and is found in the nucleus. It is possible that infection *per se*, either directly or through induction of secretion of a cytokine like tumour necrosis factor, provides the signal for this increased translocation. We may have uncovered a physiologically relevant mechanism that increases the amount of NF- κ B binding activity in the nucleus of infected cells and contributes to activation of viral transcription. That the mouse p50 precursor cannot be cleaved by the HIV-1 protease (whereas the monkey protein



infected cells by gel-shift assay. Nuclear extracts derived from uninfected CEM cells (lane 1, 3 μ g) or cells infected for 20 h (lane 2, 3 μ g) or 68 h (lane 3, 3 μ g; lane 4, 9 μ g) were assayed by gel-shift analysis using a probe covering the two tandem NF- κ B binding sites located in the HIV-1 long terminal repeat. Lanes 5-7 correspond to purified proteins which have been incubated for 1 h at 37 °C in the absence (-) or presence (+) of 60 ng purified recombinant HIV-1 protease. Lane 5, purified human p50/KBF1 (ref. 4); lane 6, purified human NF- κ B (a gift of P. Baeuerle); lane 7, purified mouse p50/KBF1 (ref. 6). Lanes 8-10 and 11-13 are with the same extracts as lanes 1-3, but using an AP1 or NF1 oligonucleotide probe, respectively. *b*, Assay of nuclear extracts from COS cells transfected with an expression vector coding for the p105 precursor followed by infection with wild-type vaccinia virus (lane 1) or a recombinant virus containing the HIV-1-derived *pol* coding region (VVG 3167; M. P. Kieny, personal communication; lane 2) using the HIV-1 NF- κ B site. Lanes 3-4 correspond to nuclear extracts derived from CEM cells either uninfected or infected for 3 days with HIV-1, respectively.

METHODS. *a*, For preparation of extracts from uninfected or infected cells see Fig. 1. Nuclear extracts (3 μ g) derived from infected or uninfected cells were assayed by band-shift as described⁶. Purified proteins were incubated for 1 h at 37 °C in double-strength binding buffer containing 0.1 M NaCl, 0.06 M Tris, pH 6.8, 2 mM DTT, 20% glycerol, 0.2 mg per ml of BSA and 0.02% NP-40, with or without 60 ng purified recombinant HIV-1 protease. This mixture was then diluted twice with water, poly(dI-dC) (30 ng) and the radioactive probe were added and incubated for 15 min at room temperature. The samples were analysed on a 5% native polyacrylamide gel in TBE buffer as described⁶. *b*, COS cells were transfected with the cDNA coding for the entire p105 precursor cloned into the RCMV expression vector (Invitrogen; V.B., P.K. and A.I., manuscript submitted). They were infected 20 h later with either wild-type or recombinant vaccinia virus containing the HIV-1 *pol* coding region (nucleotides 1,798 to 4,678, including an artificial ATG in front of the first amino acid of the protease, which is expressed by processing of the *pol* precursor) as described by Guy *et al.*¹³. Cytoplasmic and nuclear extracts were prepared 16 h after infection.

can) may also be relevant to the restricted host-range of the virus. □

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Microbial degradation of methanesulphonic acid: a missing link in the biogeochemical sulphur cycle

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ATMOSPHERIC dimethyl sulphide, arising from marine algae, cyanobacteria and salt marsh plants such as *Spartina*, is the principal sulphur compound entering the atmosphere from terrestrial and aquatic environments¹⁻⁶. Methanesulphonic acid ($\text{CH}_3\text{SO}_3\text{H}$; MSA) has been identified as a major product of the photochemical oxidation in the atmosphere of dimethyl sulphide^{1-3,5,7-9}. Dimethyl sulphide and MSA are thus predominantly, if not exclusively, biogenic in origin, and are the main gaseous links in the biogeochemical sulphur cycle. MSA is a stable compound, not undergoing photochemical decomposition³, so its removal from the atmosphere is by wet and dry deposition. MSA partitions into the aerosol phase, as well as nucleating droplet formation, and is deposited in rain and snow. Analysis of Antarctic ice cores¹⁰ gives evidence of its global deposition over many thousands of years. The subsequent fate of MSA deposited on land was unknown. Here we describe terrestrial bacteria that grow on MSA. Their activities in the natural environment would result in the mineralization of MSA to carbon dioxide and sulphate, thus completing our understanding of this part of the sulphur cycle.

Inoculating soil and water samples into media containing MSA as the sole carbon source did not give rise to organisms able to grow on MSA. Several culture collection strains, including *Pseudomonas* MS, *Bacillus* PM6, *Methylophilus methylotrophus* and *Thiobacillus versutus*, were also tested but were unable to use MSA as a carbon source. We then decided to use a chemostat-enrichment procedure with both methylammonium and MSA. This would enhance recovery of methylotrophic bacteria that might use MSA as a sole- or cosubstrate, and would also allow enrichment of specialist MSA-users unable to use methylammonium, assuming that these could compete successfully with other organisms. Such procedures have been successful in isolation and competition experiments with sulphur-using bacteria^{11,12}, and are believed to be able to enrich for several types of bacteria that can degrade MSA. Soil (100 g) from the University of Warwick campus was inoculated into a one-litre fermenter containing minimal medium E¹³ with 10 mM MSA and 20 mM methylammonium chloride (MMA). After batch culture for 2 weeks at 30 °C (stirring, 500 r.p.m.; aeration, 600 ml air per min), the culture was given an intermittent supply of new medium (flow for 1 h at a dilution rate of 0.03 h⁻¹, 2 h off) with 7 mM MSA and 3 mM MMA. Continuous flow of medium containing 15 mM MSA alone was then started. After 3 weeks, samples were streaked onto 1.5% (w/v) agar medium, containing 15 mM MSA. Organisms showing seven different colony forms were subcultured from these plates. Nine cultures were obtained (M1-M9), six of which were Gram-negative rods (including a pink-pigmented facultative methylotroph), and three Gram-positive strains: a slender rod (M5), a normal rod (M3) and a coccus (M8).

A diversity of organisms able to grow using MSA as sole energy substrate was thus present in a randomly chosen soil sample to which the only known input of MSA or other one-carbon compounds was from natural sources.

One Gram-negative strain (M2) was studied further. It grew aerobically in batch culture on MSA (10–20 mM) with a doubling

time of 7–8 h. Formate (~1 mM) was detected during growth, and there was a decrease in pH, consistent with the formation of sulphuric acid. Strain M2 showed similar growth rates on MSA and mono-, di-, and trimethylamines (MMA, DMA and TMA), and grew rapidly on methanol. In MSA-limited chemostat culture, the maximum specific growth rate of M2 was 0.091 h⁻¹, and the biomass concentration was doubled by doubling the input concentration of MSA, proving dependence on MSA. Growth on MSA produced about 10 g dry biomass per g mol. Yields on MSA and MMA were similar, and on DMA and TMA were roughly two and three times greater, respectively. It also grew aerobically on formaldehyde, formate, methyl nitrite, acetate, propionate, pyruvate, lactate, succinate, glutamate, serine and glucose, but not on methane, thiosulphate, methane phosphonate, dimethyl sulphide or dimethyl disulphide. Strain M2 is thus a facultatively heterotrophic methylotroph.

We tested the ability of MSA-grown organisms to oxidize MSA in the oxygen electrode cell by centrifuging and resuspending them in substrate-free medium. Rapid oxidation occurred until 1.5 mol oxygen were taken up per mol MSA; the rate then declined to about 7% of that seen initially. Total oxygen consumption did not reach the 2:1 stoichiometry required for complete oxidation of MSA to carbon dioxide and sulphate, but indicated that formate was accumulated under the conditions of the experiments. The oxygen: substrate molar stoichiometries for methanol, formaldehyde and formate in the oxygen electrode were 1.0, 0.5 and 0, respectively, consistent with oxidation of the former compounds only to formate. The Michaelis constant (K_m) values (substrate concentration for half-maximal oxidation rates) for MSA and formaldehyde was estimated as 0.02 and 0.08 mM, respectively. Cyanide inhibited oxidation of MSA (50% by 0.5 mM KCN) and of formaldehyde (90% by 1 mM). MSA-grown bacteria showed poor affinity for formate, consistent with formate accumulation during growth on MSA, oxidizing it only at concentrations above 16 mM.

Growth on MSA was not chemolithotrophic or autotrophic. Strain M2 could not grow on thiosulphate, so is unlikely to obtain energy from sulphonate oxidation. During growth on MSA or MMA it fixed only about 15% of its cellular carbon from [¹⁴C] carbon dioxide. The autotrophic enzyme, ribulose biphosphate carboxylase, was not detected. M2 is thus unlike *T. versutus* and *T. thioparus*, which use inorganic sulphur compounds for energy and grow autotrophically using compounds like MMA or methylated sulphides as energy sources^{5,14}.

Cell-free extracts contained no hexulose phosphate synthase^{14,15}, which was easily detected in *Methylococcus capsulatus*, assayed as a control. Strain M2 cannot, therefore, use the ribulose monophosphate cycle for carbon assimilation¹⁶. Hydroxypyruvate reductase¹⁴ was detected: extracts showed hydroxypyruvate-dependent NADPH oxidation activities of 589 nmol NADPH per min per mg protein, but no activity with NADH. We conclude that carbon assimilation by strain M2 is by the serine pathway for assimilation of formaldehyde and carbon dioxide¹⁶.

MSA degradation by strain M2 does not involve hydrolysis to produce methane, and is thus not analogous to the C—P cleavage of methane phosphonate seen in *P. testosteroni*¹⁷. Initial cleavage of MSA may be by: (1) formation of methyl-tetrahydrofolate and sulphite, analogous to *Pseudomonas* MS growing on trimethylsulphonium chloride^{18,19}; (2) attack by an MSA-specific mono-oxygenase to produce formaldehyde and sulphite, as for the NADH- and oxygen-dependent cleavage of C₄–C₁₂ *n*-alkane-1-sulphonates by *Pseudomonas*^{20,21}; or (3) hydrolysis to yield methanol and sulphite.

Clearly many bacterial types can degrade MSA, probably through diverse routes and at different rates. Such bacteria help to complete the earth-atmosphere-earth cycle of DMS → MSA → CO₂ + sulphate → DMS, and will doubtless be shown to be ubiquitous in the natural environment. □

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Ribozyme recognition of RNA by tertiary interactions with specific ribose 2'-OH groups

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SHORTENED forms of the group I intron from *Tetrahymena* catalyse sequence-specific cleavage of exogenous oligonucleotide substrates^{1,2}. The association between RNA enzyme (ribozyme) and substrate is mediated by pairing between an internal guide sequence on the ribozyme and a complementary sequence on the substrate^{1,3,4}. RNA substrates and cleavage products associate with a binding energy greater than that of base-pairing by ~4 kcal·mol⁻¹ (at 42°C), whereas DNA associates with an energy around that expected for base-pairing^{5–9}. It has been proposed that the difference in binding affinity is due to specific 2'-OH groups on an RNA substrate forming stabilizing tertiary interactions with the core of the ribozyme, or that the RNA-RNA helix formed upon association of an RNA substrate and the ribozyme might be more stable than an RNA-DNA helix of the same sequence⁶. To differentiate between these two models, chimaeric oligonucleotides containing deoxynucleotide residues at successive positions along the chain were synthesized, and their equilibrium binding constants for association with the ribozyme were measured directly by a new gel electrophoresis technique⁵. We report here that most of the extra binding energy can be accounted for by discrete RNA-ribozyme interactions, the 2'-OH group on the sugar residue three nucleotides from the cleavage site contributing the most interaction energy. Thus, in addition to the well documented binding of RNA to RNA by base-pairing^{10–14}, 2'-OH groups within a duplex can also mediate association between RNA molecules.

The L-21 *ScaI* form of the *Tetrahymena* ribozyme (E) catalyses the endonucleolytic cleavage of the oligonucleotide substrate pGGCCUCUA₅ by guanosine, giving products pGGCCUCU (matched product, or MP) and GA₅ (ref. 2). The product pGGCCUCU binds to the ribozyme with an equilibrium dissociation constant ($K_d = [E][MP]/[E \cdot MP]$) very similar to that of the substrate^{5,7}. The product rather than the substrate oligonucleotide was used in this study to facilitate

synthesis and eliminate the possibility of cleavage by ribozyme-catalysed hydrolysis during binding assays.

Chimaeric product oligonucleotides were synthesized containing single deoxyribose residues at successive positions from the 3' end of the chain and were tested for binding affinity to the L-21 *ScaI* ribozyme. The data fitted the theoretical curve for binding of a single oligonucleotide to a single molecule of ribozyme (Fig. 1). Deoxyribose substitution at position 3 (GGCCd(U)CU), three nucleotides from the ribozyme cleavage site, had the most deleterious effect on binding, followed by position 2. Substitution at position 1, 4 or 5 had no observable effect on binding. The all-RNA product bound to the ribozyme with 4.1 kcal·mol⁻¹ extra binding energy (beyond that calculated for simple RNA-RNA base-pairing; see legend to Table 1). Removal of the 2'-OH at position 3 eliminated 1.6 kcal·mol⁻¹ (39%) of this extra binding energy (Table 1). The effects of the single deoxyribose substitutions add up to 56% of the 4.1 kcal·mol⁻¹. The differential effect of individual 2'-OH groups is not unique to the 10 mM Mg²⁺ binding condition.

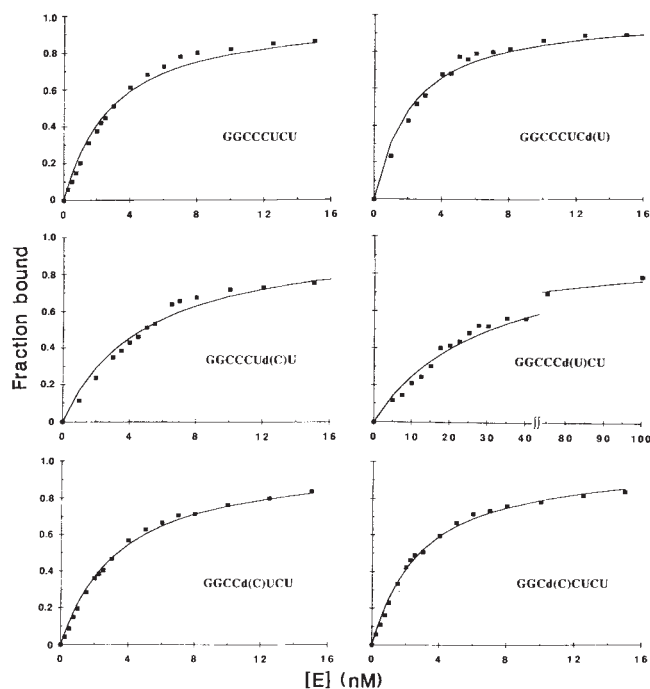


FIG. 1 Equilibrium binding curves representing association of chimaeric oligonucleotides with the *Tetrahymena* ribozyme at 42°C and 5 mM Mg²⁺. Binding was measured using the gel-mobility shift method described previously⁵. Note that the binding curve for GGCCd(U)CU has a different x-axis scale than the others. The solid line represents the fit to a theoretical binding curve. All curves were fitted to 100% final binding. The K_d for each oligonucleotide was obtained by solving a nonlinear least-squares equation describing the best fit of the data to the theoretical lines shown.

METHODS. GGCCUCU and L-21 *ScaI* ribozyme were prepared by transcription using T7 RNA polymerase². Chimaeric oligonucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer as described in the literature^{28,29} using phosphoramidites (Milligen Biosearch and ABN). Oligonucleotides were labelled at their 5' end with ³²P and purified by PAGE. The synthetic oligonucleotides ran as single bands on a gel, with electrophoretic mobilities comparable with that of transcribed RNA of the same composition. The presence of 2'-5' linkages and residual protecting groups on the bases or 2'-OH groups was ruled out by the completeness of digestion with ribonucleases T2 and A. Synthetically prepared oligonucleotides, like those prepared by transcription, bound to >90% in all cases and gave good fit to the theoretical binding curve ($S_y < 7.5\%$, S_y , standard error of estimate as described previously⁵). The electrophoresis buffer used for the binding experiments was 34.5 mM Tris, 65.5 mM HEPES, 0.1 mM disodium-EDTA and 5 mM MgCl₂, a solution which is pH 7.5 without any adjustment. This was previously termed TH₁₀₀E_{0.1}Mg₅pH 7.5.

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TABLE 1 Contribution of individual 2'-OH groups to tertiary interaction energy

Name	Oligonucleotide	K_d (nM)*	$\Delta G_{\text{obs}}^{\dagger}$ (kcal mol ⁻¹)	$\Delta G_{(2'\text{-OH})}^{\ddagger}$ (kcal mol ⁻¹)	Percentage of extra binding energy§
MP	GGCCCUU	0.8	-13.1	—	—
MPd1	GGCCCUd(U)	0.8	-13.1	0	0
MPd2	GGCCCUd(C)U	1.9	-12.5	-0.6	15
MPd3	GGCCCUd(U)CU	10	-11.5	-1.6	39
MPd4	GGCCd(C)UCU	0.9	-13.0	-0.1	2
MPd5	GGCd(C)UCU	0.8	-13.1	0	0
MPd1, 2	GGCCCUd(CU)	5.7	-11.8	-1.3	32
MPd2, 3	GGCCCUd(UC)U	58	-10.4	-2.7	66
MPd3, 4	GGCCd(CU)CU	12	-11.4	-1.7	41
MPd4, 5	GGCd(CC)UCU	2.8	-12.3	-0.8	20
MPd5, 6	GGd(CC)UCU	2.7	-12.3	-0.8	20
MPd2, 3, 4	GGCCd(CUC)U	130	-9.9	-3.2	78
MP	d(GGCCCUU)	2,100	-8.2	—	—
dMPPr3	d[GGCCCr(U)CU]	85	-10.2	-2.0¶	41
dMPPr2, 3	d[GGCCCr(UC)U]	7	-11.7	-3.5¶	71

* Equilibrium binding was measured at 42 °C and 10 mM Mg²⁺ using conditions and procedure described previously⁵. The fractional error for the values in this column is $\pm 20\%$ ($N=3$, 95% confidence limits). This value was obtained from three independent binding curves for the oligonucleotide MPd1. All K_d values were determined at least twice.

$\dagger \Delta G_{\text{obs}}^{\dagger} = R \times T \times \ln(1/K_d)$ where $R = 0.00198$ kcal mol⁻¹ K⁻¹ and $T = 315$ K (42 °C).

$\ddagger \Delta G_{(2'\text{-OH})}^{\ddagger}$: The stability conferred by 2'-OH groups(s) at the residue(s) italicized in 'Oligonucleotide' column. Obtained as follows: $\Delta G_{(2'\text{-OH})}^{\ddagger} = -13.1$ kcal mol⁻¹ - $\Delta G_{\text{obs}}^{\dagger}$ for all MPd(N) oligonucleotides.

§ The percentage extra binding energy is $\Delta G_{(2'\text{-OH})}^{\ddagger} / \Delta G_{\text{extra}}^{\ddagger} \times 100$, where $\Delta G_{\text{extra}}^{\ddagger} = -4.1$ kcal mol⁻¹ for all MPd(N) oligonucleotides, or -4.9 kcal mol⁻¹ for dMP, dMPPr3 and dMPPr2,3 since these oligonucleotides are primarily DNA in composition. The value -4.1 kcal mol⁻¹ was determined by subtraction of the calculated value of $\Delta G_{\text{bp}}^{\circ}$ (-9.0 kcal mol⁻¹ at 42 °C)⁵ from the observed $\Delta G_{\text{obs}}^{\dagger}$ for GGCCCUU (-13.1 kcal mol⁻¹ at 42 °C). (Preliminary direct measurement of $\Delta G_{\text{bp}}^{\circ}$ (42 °C, 10 mM Mg²⁺) for MP binding to the oligonucleotide GGAGGG yielded values only 0.5 kcal mol⁻¹ greater than the calculated value. An error of 0.5 kcal mol⁻¹ in $\Delta G_{\text{bp}}^{\circ}$ would give a 13% systematic error in the values for percentage extra binding energy, which provides an estimate of the uncertainty of these values (39% extra binding energy is $39 \pm 5\%$ and 15% extra binding energy is $15 \pm 2\%$.) The value -4.9 kcal mol⁻¹ was determined by subtraction of the $\Delta G_{\text{obs}}^{\dagger}$ for dMP (-8.2 kcal mol⁻¹) from $\Delta G_{\text{obs}}^{\dagger}$ of MP (-13.1 kcal mol⁻¹).

¶ This oligonucleotide is actually d(GGCCCUd(U), having deoxyribose residues at both 3' and 5' termini. Note that the G residues do not base-pair to the internal guide sequence (Fig. 2).

¶ For oligonucleotides containing primarily deoxynucleotide residues, calculated as $\Delta G_{(2'\text{-OH})}^{\ddagger} = \Delta G_{\text{obs}}^{\dagger} - (-8.2$ kcal mol⁻¹).

Binding is about three times weaker at 5 mM Mg²⁺, but the relative trend in association as a function of deoxynucleotide substituent is the same at both Mg²⁺ concentrations (Fig. 2).

To determine if cooperativity between 2'-OH groups is involved in the extra energy of RNA binding to the ribozyme, double deoxyribose substitutions were made along the oligonucleotide chain (Table 1). At positions 4 and 5, which gave no effect when singly substituted, the binding was weakened three times by the double substitution (0.8 kcal mol⁻¹), which we attribute to an effect on duplex stability. (DNA-RNA helices can be less stable than their RNA-RNA counterparts^{15,16}. Preliminary measurements of K_d (42 °C, 10 mM Mg²⁺) for MPd2,3 and MPd4,5 binding to the oligonucleotide GGAGGG, which has the same sequence as the internal guide sequence of the ribozyme, showed binding to be weakened three or four times relative to binding of MP.) The double substitution with the greatest effect was GGCCCUd(UC)U, which eliminated 2.7 kcal mol⁻¹ of the extra interaction energy. This is equivalent (within error) to the sum of the energies of the two single substitutions at these same positions ($0.6 + 1.6$ kcal mol⁻¹), plus the 0.8 kcal mol⁻¹ effect of a double substitution on duplex stability ($0.6 + 1.6 + 0.8 = 3.0$ kcal mol⁻¹). This confirms the special contribution of the 2'-OH groups at positions 2 and 3 and indicates that they act independently in binding to the ribozyme. When the effects of double substitutions are added, they account for essentially all of the calculated extra binding energy (for example MPd1,2 + MPd3,4 + MPd5,6 = 93%; Table 1).

To test further whether 2'-OH groups at positions 2 and 3 can account for most of the tertiary interaction energy with the ribozyme, DNA oligonucleotides were synthesized which contained ribose residues at position 3 (dMPPr3) and at positions 2 and 3 (dMPPr2,3). DNA binds weakly to the ribozyme, having an affinity slightly weaker than that measured for the simple

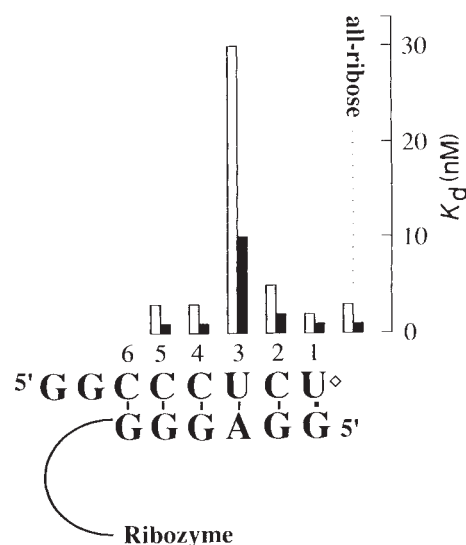


FIG. 2 Changes in oligonucleotide binding upon substitution of each individual position with a deoxyribose moiety. Open bars, 5 mM Mg²⁺. Filled bars, 10 mM Mg²⁺. K_d , the dissociation constant of the indicated chimaeric oligonucleotide from the ribozyme. The diamond shows the position of the phosphate at the reaction site, which would be present in the substrate; the oligonucleotides used in this study correspond to reaction products and have 3'-OH termini at this position. Although nucleotides preceding the cleavage site are normally indicated with negative numbers, we have omitted the minus signs here for simplicity. Note the data for all-ribose GGCCCUU to the right of the chimaeric data. Phylogenetic comparisons of group I introns^{3,30} and mutational studies on the *Tetrahymena* intron^{1,4,31} show that base-pairing between the ribozyme internal guide sequence (GGAGGG) and oligonucleotide substrate is an important component of binding. This is illustrated in the lower part of the figure, beneath the histogram.

RNA·RNA duplex (Table 1). When ribose residues are incorporated at positions 2 and 3, there is substantial (71%) restoration of binding affinity. The binding energy not restored by this double ribose substitution (29% of the total, ninefold in K_d) can be attributed to the lower stability of an RNA·DNA duplex compared with an RNA·RNA duplex, as the dMP_{2,3} oligonucleotide is primarily DNA in composition.

The compensatory increases in binding observed with dMP_{2,3} and dMP_{2,3} are comparable in magnitude to the deleterious effects observed with MP_{2,3} and MP_{2,3}. The extra interaction energy attributable to the 2'-OH at position 3 is almost the same calculated from MP_{2,3} (39%) as from dMP_{2,3} (41%). The extra interaction energy attributable to the 2'-OH at positions 2+3 is almost the same calculated from MP_{2,3} (66%) as from dMP_{2,3} (71%). Thus, regardless of the context (primarily ribose or primarily deoxyribose), positions 2 and 3 together account for most of the extra binding energy. This specific mirroring of binding effects is evidence for the presence of a tertiary interaction with positions 2 and 3 on the RNA product oligonucleotide.

Previous proposals of a tertiary interaction were based on comparisons of RNA and DNA binding to the ribozyme⁶ or comparisons of observed and calculated binding free energies^{5,7}. We have shown that the extra binding energy observed is the result of a specific interaction and that certain positions on the substrate are more important than others in contributing to the magnitude of ground-state interaction energy. The 2'-OH group on the phylogenetically conserved uridine residue at the cleavage site (position 1) does not contribute to ground-state stabilization, a surprising result considering the large contribution of this hydroxyl group to transition state stabilization (D. Herschlag, unpublished results). Influence of ribose position decreases in the order 3 > 2 > 1, 4, 5, 6. Therefore, the substrate-ribozyme complex is probably stabilized by bonds between 2'-OH residues at sugar positions 2 and 3 and residues within the ribozyme core (Fig. 3).

The effect of deoxyribonucleotide substitution was previously

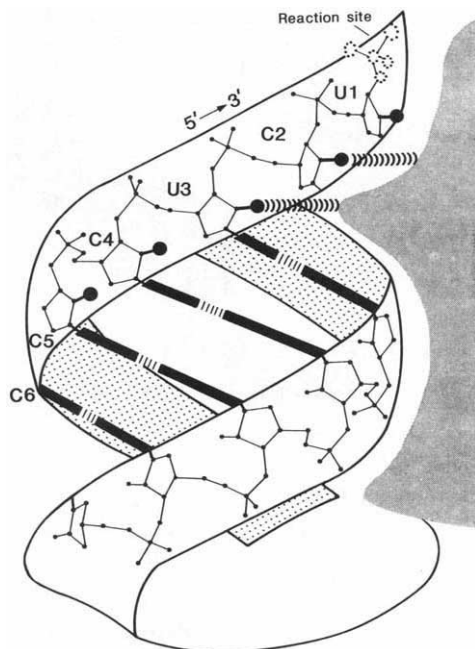


FIG. 3 Schematic of the tertiary interaction between 2'-OH groups on GGCCUCU and undefined residues within the catalytic core of the ribozyme (dark shaded region). The helix formed by binding of GGCCUCU to the guide sequence of the enzyme is called P1. Possible hydrogen bonds are indicated as '---'. The phosphate at the reaction site (dotted) would be present in this position. The oligonucleotides used in this study have 3'-OH termini at this position.

examined for a non-enzymatic reaction of the *Tetrahymena* intron: the reversal of cyclization by attack of the dinucleotide CpU (ref. 9). On the basis of kinetic measurements, Sugimoto *et al.*⁹ concluded that the 2'-OH of the C (position 2 in our nomenclature) increased the binding of the dinucleotide, and they postulated a 2'-OH-intron interaction. Because different substrate binding sites are involved in reverse cyclization and the endonuclease reaction studied here^{4,17}, there may be corresponding differences in the location of stabilizing tertiary interactions. Thus, while it is quite possible that the interaction proposed for position 2 by Sugimoto *et al.*⁹ corresponds to either the position 2 or position 3 interaction proposed here, the relationship between these two systems remains to be established.

The magnitude of the interaction energy we observe (2.7–3.5 kcal mol⁻¹ for the double substitution at position 2+3; Table 1) suggests that it could result from two or more hydrogen bonds between the oligonucleotide and enzyme^{18,19}. A 2'-OH could act either as an H-bond donor or acceptor, and could interact with the enzyme either directly or through an intermediary Mg²⁺ or water molecule. Intramolecular hydrogen bonds between ribose 2'-OH groups and nucleotide bases have been observed in the crystal structure of transfer RNA (N1 and N7 of adenine, and N2 and N7 of guanine)²⁰ and in one case the interaction has been shown to affect tRNA function on the ribosome²¹. There is also crystallographic evidence for such a linkage between the termini of two RNA duplexes (O2 of uridine)²². Furanose 2'-OH groups line the minor groove of double-stranded RNA, so the ribozyme core elements that recognize 2'-OH groups probably abut the minor groove of helix P1 on a single face (Fig. 3).

The intermolecular interaction between the *Tetrahymena* ribozyme and RNA di- and oligonucleotides provides the first example of 2'-OH groups within an RNA duplex being involved in tertiary interactions. It also emphasizes the role of the 2'-OH as a general recognition and binding determinant in RNA. The 2'-OH group on the guanosine substrate may also be involved in stabilizing its interaction with the guanosine binding site in group I introns²³. In the case of the hammerhead ribozyme, incorporation of deoxynucleotide residues in the substrate decreased the rate of cleavage^{24–26}. It remains to be determined whether any particular substrate 2'-OH group is directly involved in binding to this ribozyme.

Although a specific interaction between the *Tetrahymena* ribozyme and the sugar-phosphate backbone of an RNA oligonucleotide has been established, the functional groups in the ribozyme core responsible for this interaction remain to be identified. A theoretical model has recently been proposed in which the ribose sugars at positions 2 and 3 form hydrogen bonds with specific nucleotides in the catalytic core²⁷. We have prepared mutants of these and other conserved core residues and are screening them for their ability to bind RNA and chimaeric oligonucleotide substrates. Identification of both substrate and ribozyme contact points will provide a better understanding of ribozyme-RNA recognition. □

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Synthesis from DNA of a molecule with the connectivity of a cube

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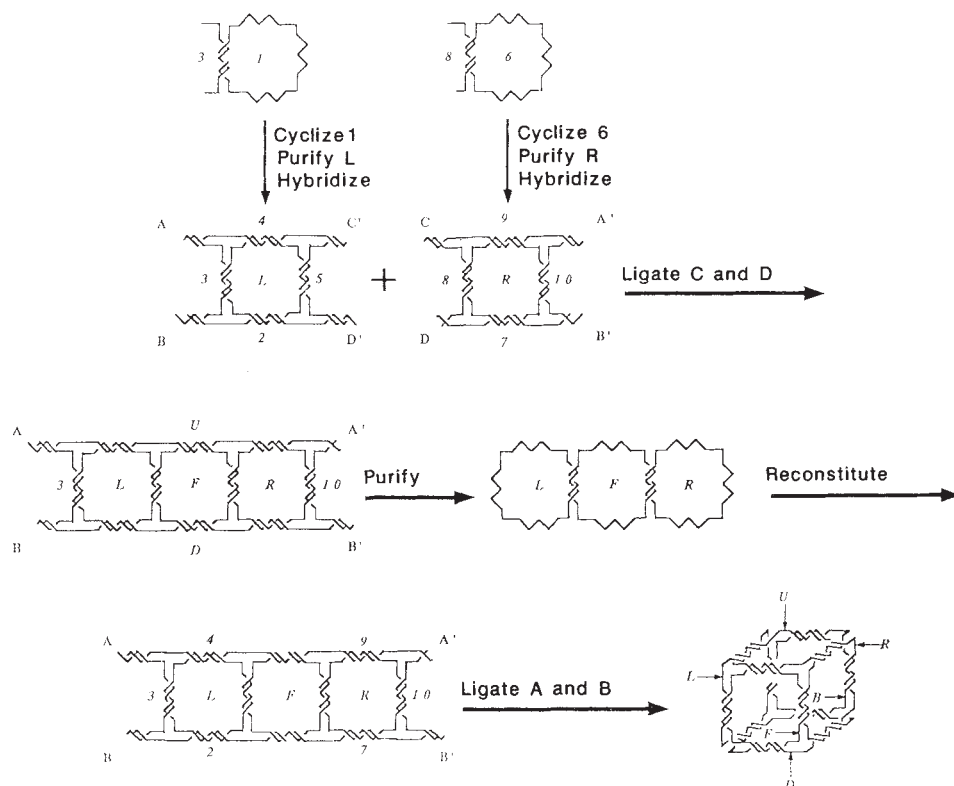
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A PRINCIPAL goal of biotechnology is the assembly of novel biomaterials for analytical, industrial and therapeutic purposes. The advent of stable immobile nucleic acid branched junctions¹⁻⁴ makes DNA a good candidate for building frameworks to which proteins or other functional molecules can be attached and thereby juxtaposed⁵⁻⁷. The addition of single-stranded 'sticky' ends⁸ to branched DNA molecules converts them into macromolecular

valence clusters that can be ligated together¹. The edges of these frameworks are double-helical DNA, and the vertices correspond to the branch points of junctions. Here, we report the construction from DNA of a covalently closed cube-like molecular complex containing twelve equal-length double-helical edges arranged about eight vertices. Each of the six 'faces' of the object is a single-stranded cyclic molecule, doubly catenated to four neighbouring strands, and each vertex is connected by an edge to three others. Each edge contains a unique restriction site for analytical purposes. This is the first construction of a closed polyhedral object from DNA.

The synthetic scheme, shown in Fig. 1, is to synthesize ten strands, corresponding to two squares, and then to ligate them together. This scheme permits phosphorylation at three stages, so that intermediate products of a well-defined nature can be analysed: (1) the 80-mer circles, strands 1 and 6 are phosphorylated, cyclized by ligation, and then associated with the other strands that make up their squares; (2) the two squares are

FIG. 1 The synthetic scheme used to synthesize the cube-like object. This diagram illustrates the strand identification used in the text. Numbers refer to strand numbers. We have used the convention that as a new strand is formed by ligation, its identification changes from one or more numbers to a letter corresponding to its position in the final object. For example, strand 1 is synthesized as a linear molecule, but is referred to as L once it is cyclized. The six final strands in the object are referred to as L (left), R (right), U (up), D (down), F (front) and B (back). Depiction of twisting between strands is confined to the central portion of each edge for clarity, following a previous convention⁷. This scheme depicts 5 steps (separating 6 stages) in the synthesis of the three-dimensional, 3-connected DNA object, after individual strands have been purified. The first step has two parts, the cyclization of the full-length strands 1 and 6 to form L and R, respectively. These two cycles are then hybridized with strands 2-5 and 7-10, respectively, to form the constituent squares shown in the second step. These squares are ligated together at the complementary sticky ends C' and C (5 base 5' overlap) and D and D' (4 base 3' overlap). This reaction forms strands U, F and D. U and D are discarded in a purification on a denaturing gel that isolates the L-F-R triple complex. The L-F-R-2-3-4-7-9-10 complex is then reconstituted and the final ligations are performed to close sites A and A' (4 base 5' overlap) and B and B' (5 base 3' overlap), as well as to seal the nicks in U (4-9) and D (2-7). This series of reactions forms the entire 3-D, 3-connected¹⁴ object. Note that each edge is doubly linked to each of its four neighbouring strands. Denoting an edge by the two strands that comprise it, the restriction sites are: LF, *DdeI*; FU, *BstEII*; RU, *Sau96I*; BU, *BstNI*; LF, *RsaI*; FR, *BstUI*; RB, *HhaI*; BL, *AluI*; FD, *HinfI*; RD, *TaqI*; BD, *StyI*; LD, *HaeIII*. The sequences of the individual strands are: strand 1: CTGCTCATACTGCA-



TTCCGCCAGCCTGACATCACCGTGTACGCCCAAACCTTTCAACTTAGATGGTAGA-AGGAGGGCAG; strand 2: CGCTGTGGGTACGGCTGGCCGAATGCAGAGCCAAT-CCTTGG; strand 3: GATTGGCTTATGAGCAAGCTGCCCTCCTCGTTAGTT; strand 4: CTGGAACTAACGTCTACCATCTAAGTTGAAAGTCTCTTG; strand 5: GTGAC-CAAGAGAGTTTGGCGGTACACGGTGTATCCACAGCGACTC; strand 6: CGTGCTA-ACAGGTAGAGTTTCGACGAATTACACAAATCGGCGCAATACTATCCCGACTTGA-CCAGCCTTTCGCCATCTCG; strand 7: GTGATTGGTAATTCGTGCAACTCTACC-CTGAATGCGAGT; strand 8: GCATTTCAGTGTATAGCACGCGAGATGGCGTTCTGACG; strand 9: GTCACCGTCAGAAAAAGGCTGGTCCAAGTCGGGGCAGCGTC; strand 10: CCAGGACGCTGCATAGTATTGCGCCGATTGTCAATACCCCAAG.

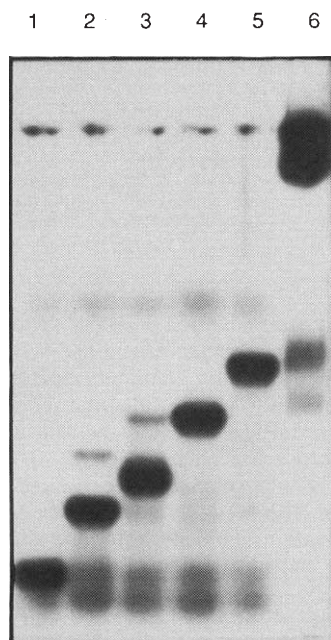


FIG. 2 The association of individual strands 2-5 with strand L to form a quadrilateral. Autoradiogram of a native gel in which strand L has been labelled and cyclized previous to the reactions shown. The first five lanes shown indicate the sequential addition of strands 2-5 to strand L. Lane 1: strand L; lane 2: L + 2; lane 3: L + 2 + 3; lane 4: L + 2 + 3 + 4; lane 5: L + 2 + 3 + 4 + 5. Lane 6 shows the ligation of two quadrilaterals to form the belt-like structure shown in Fig. 1, following ligation of ends C and D. The dark band near the top of the well in lane 6 contains the belt-like intermediate and many failure products as well, indicating that one cannot separate the desired product by native gel electrophoresis. The topmost band (top of the well) contains a complex of smaller species that cannot penetrate the gel. The DNA molecules used are synthesized on an Applied Biosystems 380B automatic DNA synthesizer, removed from the support and deprotected using routine phosphoramidite procedures¹⁵. DNA molecules are purified from denaturing gels. Hybridized complexes are formed by mixing a stoichiometric quantity of each strand, as estimated by absorbance at 260 nm; this mixture is heated to 90°C for 5 min and slowly cooled. Errors in stoichiometry are corrected by electrophoresing the complex on a non-denaturing gel, and extracting the appropriate band from the gel. Except where noted in the text, all gels contain 6% polyacrylamide (19:1, acrylamide:bisacrylamide).

annealed with only those phosphates on the end-labelled C, C', D and D'; (3) the last phosphorylation before reconstitution puts a 5' phosphate on all unphosphorylated strands, and is followed by the final ligation. The sequences of the individual strands (see legend to Fig. 1) have been assigned using the program SEQUIN (ref. 9). Each square contains no repeated contiguous stretch of 6 nucleotides^{1,9}. Only 28 of 480 such sequences are duplicated in the entire cube. In principle, only two of the steps used are necessary for the synthesis of the cube: (1) ligation of C and D, simultaneously with the covalent cyclization of strands 1 and 6, followed by (2) the ligation of A and B. The five steps shown in Fig. 1 are a response to the failure of that approach.

Figure 1 shows the strand numbering and nomenclature used. The association of L with strands 2-5 is shown in Fig. 2; similar results are obtained with R and strands 7-10. We show that these fifth-order reactions go largely to completion, but this does not guarantee that all strands are double-helical. For example,

strand 2 might be paired with strands 3 and 5, but not with L. Both here and in the reconstitution steps below, we use susceptibility to restriction endonucleases as an assay for perfect pairing, as most of our sites are recognized by enzymes with exclusively double-helical activity. In each case, complete digestion indicates that the relevant strands have completely hybridized.

Ligation of square L to square R forms the tricyclic belt, shown at the third stage of Fig. 1. The desired product of the ligation is not separable from other products on non-denaturing gels (Fig. 2), but successful double ligation of cohesive ends C and D is identified by a 3-circle catenated intermediate (L-F-R) after treatment with exonuclease III. Figure 3 shows a denaturing gel with numerous exonuclease III-resistant product bands following ligation of C and D. Figure 3 also shows the products obtained when 21 nucleotide pairs per edge are used, rather than 20; the L-F-R band is virtually absent. We purify the catenated L-F-R core of the belt on denaturing gels, and then reconstitute it by the adding the missing six strands, now phos-

FIG. 3 The products of the first ligation (ends C and D). Autoradiogram of a denaturing gel illustrating the products of ligating two quadrilaterals together at ends C and D. Kinase labelling and ligations were as described⁷. The quadrilaterals are radioactively labelled on the F strand. The first two lanes illustrate the ligation of quadrilaterals containing 20 nucleotide pairs per edge, whereas the quadrilaterals used for the second two lanes contain 21 nucleotide pairs per edge. Lanes 1 and 3 show all the products, and lanes 2 and 4 show the products following digestion with exonuclease III. Small products seen in these lanes result from incomplete digestion. The symbols on the left and right indicate the total number of nucleotides in the species in the band with the indicated mobility. L means linear species that degrade upon treatment with exonuclease III. The other symbols indicate circular or catenated species, with two linked circles indicating the L-F or F-R catenane (Fig. 1), and three linked circles denoting the L-F-R catenane (Fig. 1). Note the virtual absence of this material in the lane 4, indicating that 21 nucleotide pairs per edge are inappropriate for closing the central cycle.

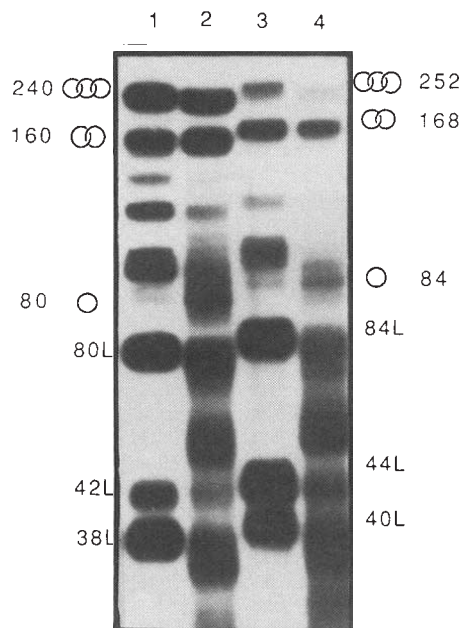
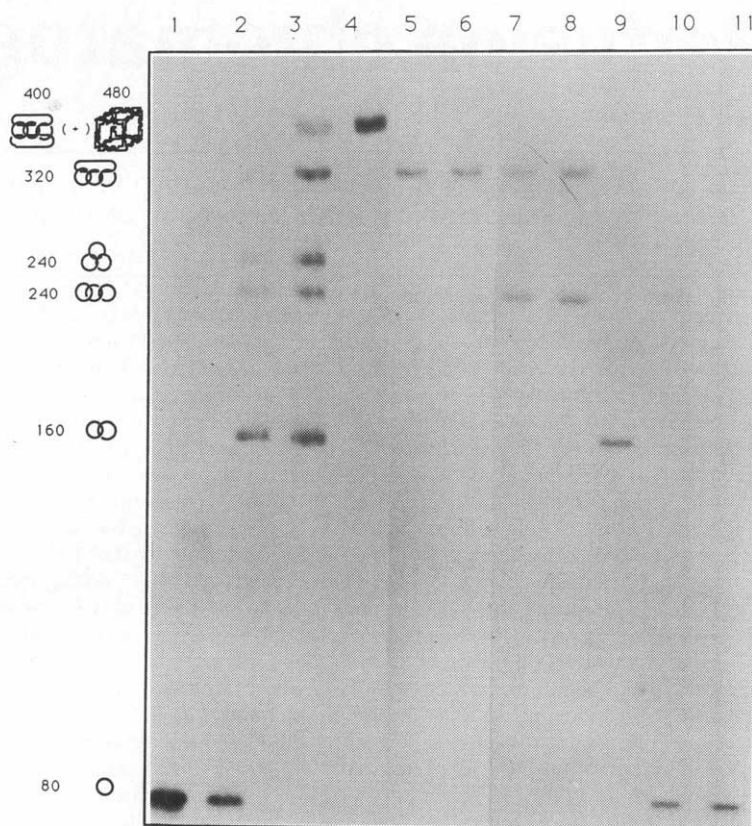


FIG. 4 Products of the final ligation step. Autoradiogram of denaturing gels showing the formation and analysis of the products of the final ligation step. The same labelling conventions apply as in Fig. 3. In addition symbols have been added to indicate other structures: Three cyclic rings correspond to a corner (for example, L-F-U (Fig. 1)); the 4-cycle symbol corresponds to a molecule with the connectivity of L-F-R-U (Fig. 1); the 5-cycle symbol corresponds to the cube lacking a single strand, for example, L-F-R-U-D (Fig. 1); the 6-cycle structure is drawn as the cube representation of Fig. 1. All material has been treated with exonuclease III. Lane 1 contains a cyclic 80-mer marker. Lane 2 contains markers (individually synthesized and then combined) corresponding to the intermediate products (up to four cycles) shown on the left. Lane 3 illustrates the products of the final ligation. The portion of the gel containing the 80-mer circle in this lane was lost during manipulation. The top most band in this lane contains a mixture of the 6-cycle final product and the 5-cycle failure product, as indicated by the (+) symbol. These materials have been separated, and lane 4 contains the purified 6-cycle final product (see text). The digestions in lanes 5-11 were all performed on this purified material, which has been labelled in strand U. Lane 5 illustrates the digestion of the product with *Bst*UI (FR) and lane 6 illustrates the digestion of the product with *Rsa*I (LF) to yield 4-cycle products. Lane 7 illustrates the double digestion of the product with *Alu*I (BL) and *Hha*I (RB) to yield a 3-cycle belt. Lane 8 illustrates the double digestion of the final product with *Bst*UI (BL) and *Rsa*I (LF) to yield another belt. Note that the presence of this belt implies the successful ligation of each bond indicated in the final step. Lane 9 shows digestion with *Alu*I (BL) and *Taq*I (RD) to yield a 2-circle catenane. Note the absence of a doublet, suggesting the absence of mixed (single and double) catenation species. Lanes 10 and 11 are a duplicate experiment in which the product has been digested with *Alu*I (BL), *Hha*I (RB), *Bst*UI (FR) and *Rsa*I (LF). The product is a single 80-mer circle; absence of 160-mer circles indicates that no detectable doubling of the belt has occurred.



phorylated. Once closed, the cube consists of six linked circles, corresponding to the six faces of the object, and labelled left (L), right (R), up (U), down (D), front (F) and back (B). Figure 4 shows a denaturing gel containing the results of the final ligation. The cube comigrates with a 5-strand standard, but can be purified on 13% denaturing gels containing 1.25% bisacrylamide. The yields from square ligation and from final closure are each about 10%.

Restriction of each edge individually results in the production of a 4-circle molecule (see for example Fig. 4, lanes 5 and 6; also data not shown). The final ligation forms the U, B and D circles. Restriction of the L-F and F-R edges results in the U-B-D triple catenane (Fig. 4, lane 8). This is the most robust proof of formation of the cube, as the belt present at the start of the last step is destroyed by restriction. Whether the final product corresponds to a single or multiple cycles of the belt (octagonal prism, dodecagonal prism, and so on) may be determined by the size of the U or D strand: at the concentrations used (10 nM in belt), the cube is the main product

(Fig. 4, lanes 10 and 11).

The side of the belt facing the reader at the branch points (Fig. 1) corresponds to the major groove of DNA, whereas the minor groove is away from the reader. We do not know on which side we have closed the belt, nor whether we have a mixture. Model building¹⁰ suggests that closure behind the page (minor groove) results in a large and more open structure. We have noted that the belt containing 20 nucleotide pairs per edge is much more readily formed than the one containing 21 nucleotide pairs per edge. This finding suggests that the twist around the F square, when attached to the L and R squares, is less than the eight cycles one would expect^{11,12}. Presumably this decreased twist occurs at the site of the junctions. Whether it is shaped like a cube or another parallelepiped, the very tight angles between the object's negatively charged edges are probably more difficult to realize than the angles that characterize better approximations to the sphere¹³. The synthesis of this object establishes that it may be feasible to make larger and more complex objects^{13,14}. □

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Perfusion chromatography

Fred E. Regnier

Perfusion chromatography is a technique based on fluid dynamics for reducing stagnant mobile phase mass transfer in liquid chromatography. This is achieved by using supports with large pores that allow mobile phase to flow through particles.

THE resolving power of liquid chromatographic systems has been improved dramatically over the past 20 years^{1,2}. Much of this success can be attributed to the minimization of band spreading through the design and production of new packing materials. Theory indicates that band spreading is predominately caused by intraparticle diffusion; longitudinal diffusion, solute diffusion in the mobile phase, eddy diffusion, and adsorption-desorption kinetics play a less significant role³⁻⁵. Diffusion through stagnant pools of mobile phase in particles is a slow process, particularly in the case of macromolecules⁶. Although this problem may be diminished by reducing particle size and, therefore, diffusion path length, it is not practical to use porous particles of less than 3–5 μm .

Recent studies have shown that there is another solution to the problem of stagnant mobile phase mass transfer. A new chromatographic packing material (POROS) has been developed, which has particle transecting pores of 6,000–8,000 Å diameter⁷ to allow liquid flow through the sorbent. The advantage of this perfusion process is that intraparticle flow convectively transports solutes to stationary phase in the particle interior. The convective transport of proteins into 10- and 20- μm particles at high mobile-phase velocity was found to be an order of magnitude more rapid than diffusive transport. POROS-based supports separated proteins in less than a minute at mobile-phase velocities 10–30-times higher than those used in high-performance liquid chromatography⁸.

This process of reducing intraparticle mass transfer by perfusing liquid through a support particle has been called 'perfusion chromatography'^{7,9}. Because perfusion chromatography is based on fluid mechanics, it is possible to enhance the kinetics of mass transfer without altering the separation mechanism. Any mode of chromatographic separation can be carried out with this perfusable packing. POROS-based media for reversed-phase, hydrophobic interaction, ion-exchange, immobilized metal affinity, and bioaffinity chromatographic separations have been prepared and used in the perfusion regime⁷.

Supports

Chromatographic supports for proteins are generally polysaccharides, polyacrylates or silica. These materials suffer from a lack of either chemical stability or mechanical

strength. Poly(styrene-divinylbenzene) (PS-DVB), by contrast, has outstanding chemical stability and is mechanically stable to 200 bar in analytical columns¹⁰. The resolution of a PS-DVB-based, reversed-phase column was unchanged after six months of operation with a 60 per cent formic acid mobile phase. Based on these properties, PS-DVB was chosen to prepare the particles used for perfusion chromatography.

The perfusable packing materials in the studies described above were fabricated in a multiple-step copolymerization of styrene and divinylbenzene. Electron microscopy shows that these particles have essentially a bimodal pore network in which the 6,000–8,000 Å through-pores are interconnected by 800–1,500 Å pores. Solute is convectively transported into the interior of the particle by the 6,000–8,000 Å through-pores and then diffuse into the 800–1,500 Å pores. The term 'diffusive pore' will be used to describe these smaller interconnecting pores. Diffusive pores should be less than 1- μm deep to minimize resistance to mass transfer at high speed. Five proteins were separated

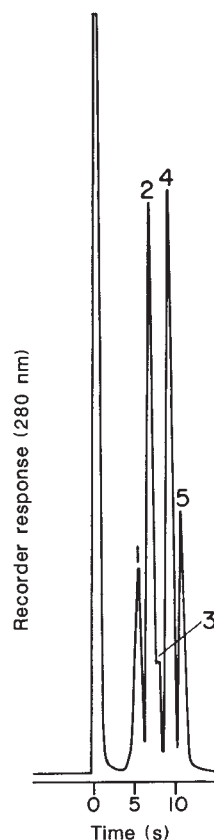
in 12 seconds (see Fig. 1) at a mobile-phase velocity of 3.6 cm sec^{-1} (ref. 8). At this flow rate, the entire liquid volume of the 68.3- μl column was displaced in 0.82 seconds.

The benefits of perfusion chromatography are greatest at high mobile-phase velocities, that is, 1,000–10,000 cm h^{-1} , where intraparticle convective transport greatly exceeds the rate of diffusive transport. Band spreading in perfusion chromatography packings will be similar to conventional high-performance packings at mobile-phase velocities of 10–100 cm h^{-1} . This is because intraparticle convective mass transport is smaller than diffusive transport at these low velocities. As mobile-phase velocity is increased to 100–1,000 cm h^{-1} , the convective and diffusive mass-transfer rates will be more or less equal. Generally, the perfusion regime calls for flow rates of greater than 1,000 cm h^{-1} , where intraparticle transport is dominated by convection. Mobile-phase velocities of 40, 400 and 4,000 cm h^{-1} are in the mid-range of operation with conventional soft gel, high-performance and perfusion chromatography columns, respectively. Because the diffusion rate of a macromolecule may be 10–100-times slower than that of small molecules, perfusion enhancement can be observed at less than 1,000 cm h^{-1} linear velocity for large proteins and greater than 1,000 cm h^{-1} for small peptides.

The design objective in this research was to obtain 10–20- μm particles with little resistance to mass transfer at mobile-phase velocities up to 3 cm sec^{-1} . This was achieved by using numerous, large through-pores and a minimum of diffusive pores. However, increasing the porosity of a support generally reduces surface area and loading capacity. The protein loading capacity of POROS-based, reversed-phase materials is approximately 50 per cent of other preparative packings. This is acceptable in analytical and semi-preparative columns, but a disadvantage for preparative work. With other surface chemistries (ion exchange, affinity), the capacity is 75–125 per cent of conventional materials. The surface chemistry can be used to compensate for a lower surface area.

Some preparative applications require a reversed-phase support with a higher loading capacity and a higher binding strength. In order to meet the demand, the pore morphology of POROS has been modified to incorporate a greater number of diffusive pores in addition to the through-pores. The loading capacity of these new sorbents is 2–4-fold

FIG. 1 A high-speed, reversed-phase separation of proteins with a steep gradient. Sample, operating parameters and peak identification were as specified in the text. Elution was achieved with a 24-second gradient. (From ref. 8.)



higher with minimal effect on kinetics. For example, the lysozyme binding capacity of this reversed-phase support is twice that of the first-generation materials. When coated, these new materials also have a higher capacity. For instance, the lysozyme loading capacity of the higher surface area strong cation exchange sorbent is 80 mg ml⁻¹.

Surface chemistry

Because poly(styrene-divinylbenzene) (PS-DVB) is a hydrocarbon, it can be used for reversed-phase chromatography without derivatization. Excellent separations of proteins, peptides and polynucleotides have been reported on PS-DVB columns^{7,9-12}. Little difference in selectivity between silica-based alkylsilane and PS-DVB reversed-phase columns has been noted for proteins.

Retention on reversed-phase chromatography columns can, however, be very different with the same mobile-phase ratio. High-porosity PS-DVB packings generally have less surface area and therefore less stationary phase than 100 and 300 Å pore diameter silica-based packings. Gradient elution will occur sooner on a high-porosity column. Adjusting either gradient slope or solvent composition compensates for this difference.

Use of the perfusable PS-DVB support in all other forms of chromatography involving polypeptides requires a tightly crosslinked, hydrophilic surface layer that masks the underlying hydrophobicity of the matrix. The surface layer used is a copolymer composed of at least three monomer species, each having a different function¹³. One of the monomers is used to anchor the copolymer to the surface of the support. This prevents erosion of the coating. The function of a second monomer is to couple stationary-phase groups to the support. Still another monomer is used to crosslink individual polymer chains into an impermeable surface layer. Both the surface copolymer and PS-DVB matrix are stable from pH 1-14.

Applications

The activities of research laboratories during the 'discovery phase' of a polypeptide have changed little over the past 30 years. The isolation and characterization of proteins and peptides can take weeks, months or even years, of which, less than one-third of the time is usually spent performing chromatographic fractionations; dialysis, buffer preparation, manual manipulations, elucidation of the structure, and structure/function studies require much more time. This would lead many protein chemists to question the need for columns that separate proteins in less than 30 seconds.

With the advent of biotechnology, this classical picture of biochemical research is changing. There can now be a 'development phase' and a 'production phase' in the history of a polypeptide. Through genetic engineering, polypeptides can now be produced on a large scale and used among a series of applications.

Where does high-speed chromatography fit in this expanded picture of biochemistry? The question is most easily addressed by examining the difference between these phases. The discovery phase is directed at elucidating polypeptide structure and properties. Analytical speed and automation are of little value in non-routine activities that are performed only once. When moving from discovery to the development and production phases, which are directed more toward optimization and confirmation, there is a shift from non-routine to more routine research activities. Maximizing production, optimizing isolation, confirming biosynthetic fidelity, structural conformation and the absence of host contaminants, collecting data for feedback control in production, examining interactions with other species, determining product shelf life, and following peptide metabolism are all activities in which speed of analysis is important. A rapid determination of IgG in a hybridoma cell supernatant is shown in figure 2.

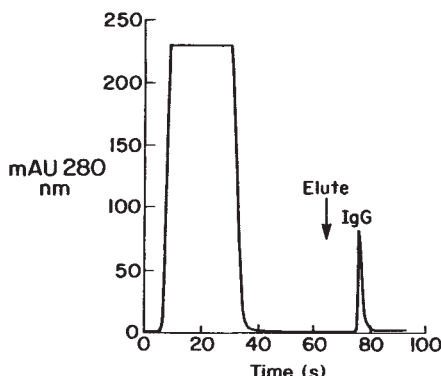


FIG. 2 Separation of IgG on Protein A. A 2.1 × 30-mm POROS A/M Protein A column was loaded with 1 ml of culture supernatant at 2 ml min⁻¹. The sample was loaded in a 0.1 M phosphate buffer (pH 7.5) with 0.15 M NaCl and eluted with 2 per cent (v/v) acetic acid with 0.3 M MgCl₂.

Perfusion chromatography is a new technique based on fluid mechanics that decreases the separation time of proteins by an order of magnitude or more. Rapid separations in all the conventional modes of chromatography, except size exclusion, have been achieved using the perfusion regime. The technique will be of greatest use in two areas: (1) an analytical environment where large numbers of routine analyses are required and (2) a process environment where enhanced intraparticle mass transport will increase throughput and productivity. □

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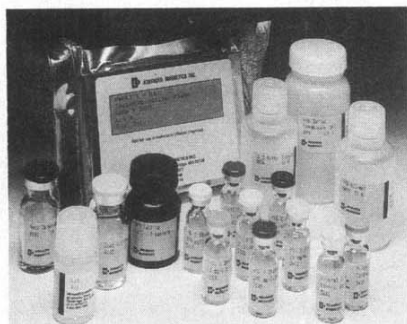
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FASEB on my mind

Over 500 exhibitors will be attending next week's FASEB (Federation of American Societies for Experimental Biology) meeting in Atlanta, Georgia, USA.

KAMIYA Biomedical has prepared four new **protein kinase inhibitors** — the KT Series — from synthetic derivatives of K252a, a microbial metabolite of *Nocardia* sp. (*Reader Service No. 101*). By replacing specific reactive groups on the general protein kinase inhibitor, Kamiya has developed the following: K-252b, KT5720, KT5823 and KT5926. According to the company, these new inhibitors have increased specificity for the following protein kinases: protein kinase C (K-252b), cyclic AMP-dependent protein kinase (KT5720), cyclic GMP-dependent protein kinase (KT5823), and myosin light-chain kinase (KT5926). KT5720, KT5823 and KT5926 cost \$300 (US) for 1 mg; K-252b costs \$280 (US) for 1 mg. See booth 1555 at FASEB.

Advanced Magnetics has developed an enzyme immunoassay (EIA) kit that is designed for the rapid **quantitative determination of human IL-2 levels** in serum or cell culture supernatants in less than three hours (*Reader Service No. 102*). The \$295 (US) IL-2 EIA kit is based on a solid-phase sandwich assay. It is supplied with well strips that are precoated with IL-2 antibody, and suffi-



IL-2 EIA kit — see booth 741.

cient prediluted, lyophilized standards and reagents to perform 96 determinations, including standard curves. Each test uses only 100 µl or less of sample, standard and control. The kit has a detection sensitivity of less than 6 pg of IL-2 per 0.1-ml sample; the sensitivity in buffer-based curves is 8.76 pg per assay 0.1-ml sample, says the company. The assay is specific for bioactive human IL-2 and recognizes both natural and recombinant forms. Stop by booth 741.

See booth 1628 for Zymed's new **monoclonal antibodies to substrates of tyrosine kinases** that are stimulated by activated epidermal growth factor receptor (*Reader Service No. 103*). These high-affinity antibodies include anti-MAP2 kinase, anti-phospholipase Cγ1, anti-paxillin, anti-anexins I and II, anti-ezrin and anti-GAP

(GTPase activating protein). Zymed says, these antibodies are suitable for western blotting, ELISA and other immunochemical techniques. They can be conjugated to a variety of signal generators, including horseradish peroxidase, fluorescein isothiocyanate, biotin and agarose.

Site-specific immobilization

BioProbe, exhibiting in booth 651, has introduced AvidPlate-HZ **surface-modified microtitre plates**, which use hydrazide chemistry to immobilize antibodies through the carbohydrate moieties of the Fc region (*Reader Service No. 104*). Unlike the random, non-covalent passive absorption of antibodies to the microplate in conventional enzyme immunoassays, AvidPlate-HZ microtitre plates provide strong directional attachment of antibodies, stable to 3 M urea and detergents, says BioProbe. In addition, as it is an Fc-specific coupling, the activity of the bound antibody is preserved; this results in increased assay sensitivity over a wide range of antigen concentrations. Flat or round-bottom AvidPlate-HZ microtitre plates are available. Each type is sold in quantities of 20 and 100 plates costing \$100 (US) and \$450 (US), respectively.

MicroBIND-HZ **covalent binding plates** from Dynatech are designed to allow site-specific immobilization of antibodies through the carbohydrate moieties of the Fc region (*Reader Service No. 105*). Consequently, the Fab regions are oriented away from the matrix, making them available for antigen binding. In addition, proteins to be immobilized do not undergo crosslinking, as the reactive group (aldehyde) does not react significantly with the primary amines of proteins at the recommended pH of 5–6, says Dynatech. This minimizes the loss of antibody activity due to crosslinking, resulting in a higher binding activity with less antibody. Unlike other covalent binding plates, crosslinking agents are not required with MicroBIND-HZ Plates, which are sold in packs of five plates or boxes of 20. See booth 1052.

Cell science

Oncogene Science has introduced two time-saving systems for **CD selection and identification** called the CytoPan Cell Selection and FluoroSort Cell Identification Systems for CD antigens (*Reader Service No. 106*). CytoPan Cell Selection Systems are offered for both positive and negative enrichment of all major lymphoid cell classes from peripheral blood or dissociated tissue samples. According to Oncogene, as little as two per cent of a heterogeneous starting population can

be recovered with a purity of over 95 per cent. Cell Selection Systems provide for panning the equivalent of ten, 100-mm diameter plates and cost \$250 (US) for T cell/B cell, myeloid cell, dendritic cell and natural killer (NK) cell systems; systems for T-cell subsets and *c-fms*/myeloid cells cost \$340 (US) and \$325 (US), respectively. FluoroSort reagents, which complement the CytoPan systems, can be used to monitor separation efficiency or to identify simultaneously two different cell populations in a single fluorescence analysis. The two different populations appear as either red or green fluorescent cells when analysed by either flow cytometry or standard fluorescence microscopy using a single filter (fluorescein). Each FluoroSort reagent is supplied pretitrated and is sufficient for 100 tests. FluoroSort cell identification reagents for T cell/B cell, T helper/T cytotoxic cell and T cell/NK cell are available, each costing \$250 (US). Oncogene Science will be in booth 1416.

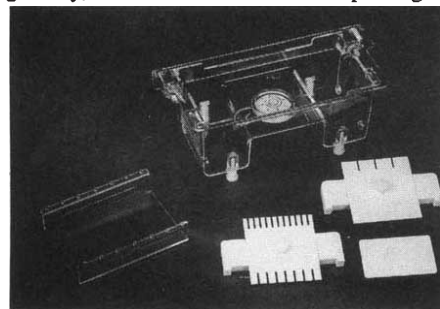
Visitors to booth 1149 can see the new Coulter CLONE Kappa-FITC and Lambda-FITC **goat polyclonal antibody reagents**, which can be used to identify and enumerate cell populations bearing kappa or lambda immunoglobulin light chains by either fluorescence microscopy of flow cytometry (*Reader Service No. 107*). These reagents should provide useful research tools for the investigation of leukaemias, lymphomas and immunodeficiency diseases, and can be integrated into standard procedures for kappa and lambda staining using mononuclear cell preparations. Whole blood can be used, provided that the blood cells are thoroughly washed to remove serum immunoglobulin, says Coulter. The company also offers goat IgG-FITC Control, which is essential for monitoring the levels of non-specific antibody Fc binding in each sample.

PharMingen has expanded its line of **conjugated monoclonal antibodies specific for murine lymphocyte cell-surface markers** (*Reader Service No. 108*). The line-up of TCR V region-specific monoclonal antibodies includes: VB3, VB5.1, 5.2, VB6, VB7, VB8.1, 8.2, VB9, VB11, VB13, VB17a, Va3.2, Va11, Vy2, Vy3 and Vδ4. In addition, the following specificities are available: IL-2 receptor, CD69, LPAM-1, LECAM-1, NK cells, heat-stable antigen (J11d) and BP1/6C3. These are available as biotin and fluorescein conjugates. Many of PharMingen's monoclonal antibodies are available as R-phycoerythrin (PE) conjugates. The company offers custom PE conjugation services. See booth 2337.

Fluorochrome-conjugated monoclonal antibodies to rat T-cell markers, which are suitable for flow cytometric analysis, are newly offered by Caltag (*Reader Service No. 109*). The range includes murine monoclonal antibodies to: rat Thy-1, available as a fluorescein isothiocyanate (FITC) conjugate (\$90 (US) for 100 tests, where one test is equal to one million cells by flow cytometry); rat CD4, available as a R-phycoerythrin (PE) conjugate (\$60 (US) for 50 tests); and rat CD8, available as both FITC (\$90 (US) for 100 tests) and R-PE conjugates (\$60 (US) for 50 tests). The FITC and R-PE conjugates allow for various combinations of two-colour analysis by flow cytometry. In addition, Caltag has a range of fluorochrome- and biotin- conjugated monoclonal antibodies to mouse cell-surface markers — see booth 2635.

Small-scale electrophoresis

With its one-piece, injection-moulded construction, the EC370 Minicell submarine gel system from E-C Apparatus is designed for leak-free use during DNA or RNA separations in agarose gels (*Reader Service No. 110*). The Minicell's UV-transparent tray with UV-fluorescent rule provides a permanent reference in gel photographs. Rubber end blocks, which slide onto the ends of the gel tray, eliminate the need for tape in gel



E-C's minicell gel apparatus.

casting. In addition, built-in levelling feet and a bull's-eye level ensure that the gel is of uniform thickness and that the buffer level is even, says E-C Apparatus. The \$195 (US) Minicell apparatus includes the main chamber, two end blocks, well former, depth and levelling gauge, UV-transparent tray, two 12-well, 8-well, 4-well and 1-well combs, two comb holders and a safety cover. The company's electrophoresis equipment will be featured in booth 1013.

Measuring 7.2 cm × 17.7 cm × 8.6 cm, the Quickee Minigel apparatus from Bios is intended to provide users with a quick check of, for example, enzyme digestions, PCR reactions, ligations and plasmid preparations (*Reader Service No. 111*). The minigel device can accommodate gels with dimensions of 0.8 cm × 7.4 cm × 5.0 cm, and requires only 26 ml of agarose, says Bios. Several handy design features have been included. Gels are cast directly in the lid of the unit or in the optional gel-casting tray. The UV-transparent running tray eliminates the need for

glass slides. Various combs are available. Bios will be in booth 2327.

Molecular miscellany

By exploiting the selective affinity of oligo-(dT)-cellulose for polyadenylated eukaryotic RNA (Poly (A)⁺ RNA), Boehringer has developed a **mRNA purification kit** that is designed to isolate high-purity yields of Poly (A)⁺ RNA from total RNA (*Reader Service No. 112*). Using a batch-loading



mRNA purification in 90 minutes.

procedure that eliminates column equilibration steps, dry oligo-(dT)-cellulose is mixed with the denatured RNA sample and the slurry is added to the column. Using this method, Boehringer claims that purified mRNA can be obtained with two passes through the column in about 90 minutes (40 minutes for the first pass, 50 minutes for the second). After two passes, 1.2–2.2 per cent of the total RNA is recovered and greater than 90 per cent of the RNA recovered is poly (A)⁺ RNA, says the company. The purified mRNA obtained is suitable for northern blot hybridization, cDNA synthesis (library construction) and *in vitro* translation. See booth 1912.

Using an 'anchor-ligated' PCR technique, the Uni-Amp Universal Amplification and Cloning System from Clontech allows for PCR amplification and cloning of small amounts of DNA of unknown sequence (*Reader Service No. 113*). Clontech says the technique is also useful for producing hybridization probes or for making cDNA libraries from small amounts of RNA. A Uni-Amp Adaptor is ligated onto both ends of blunt-ended DNA or cDNA. The DNA is then amplified by PCR using a single, complementary Uni-Amp Primer. The Uni-Amp Adaptors contain a built-in restriction site to allow for direct cloning of the amplified product, says Clontech. The \$180 (US) Uni-Amp Ligation and Amplification Kit contains Uni-Amp Adaptors and all the necessary reagents for 30 ligations. The \$450 (US) Uni-Amp Plus Kit contains all the components of the Uni-Amp Kit as well as all reagents for cDNA synthesis. Uni-Amp Adaptors containing *Eco* RI, *Sal* I, *Not* I, *Xho* I and *Xba* I are also available separately from Clontech. The Uni-Amp Primers that are required must be purchased separately. Catch Clontech in booth 2542.

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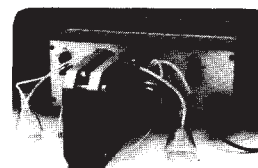
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full-length, first strand cDNA from mRNA for use in PCR (*Reader Service No. 114*). The kit uses MeHgOH (methyl mercury hydroxide) to eliminate secondary structure in mRNA, allowing AMV reverse transcriptase to produce full-length cDNA using specific oligo-dT or random primers. Template prepared using the cDNA Cycle Kit can be amplified directly without phenol extraction, ethanol precipitation or removal of the first strand primer. Invitrogen says the kit can be used for direct gene amplification of cDNA from poly (A)⁺ and non-polyadenylated RNA, verification of low-level gene expression, cDNA preparation from 1 ng to 1 µg of mRNA, and RNA denaturation to ensure full-length PCR products. Each kit contains test RNA with specific primers that can be used as an internal control for first strand synthesis and amplification. The kit also includes random and oligo-dT primers for reverse transcription, gene amplification buffer and reagents for 25 or 100 first strand synthesis reactions, costing \$175 (US) and \$375 (US), respectively. See booth 324.

What's new, what's hot

Gibco BRL has introduced a *Guide to Bacterial Transformation*, which contains details of the company's 13 varieties of frozen competent cells (*Reader Service No. 115*). The **bacterial transformation guide** should

provide researchers with useful practical information on how to choose the right efficiency and host for an application, how to perform the transformation and troubleshoot transformation protocols. The guide outlines a new transformation method called electroschock transformation, which yields greater than 1×10^{10} transformants per microgram, as well as information on how to use the new ElectroMax Cells with this technique. See booth 312.

Over 4,000 strains of yeasts are listed in the 230-page, 19th edition of the **American Type Culture Collection reference catalogue of yeasts** (*Reader Service No. 116*). Strain descriptions, which include genotypes, source of isolation, literature references, media formulae and special applica-



Yeast strain reference guide.

tions, are included in the catalogue. A new section contains chromosome maps of overlapping continuous genomic clones (contigs) from *Saccharomyces cerevisiae*. The catalogue is available free of charge in the USA; it costs \$5 (US) in Canada and Mexico, and \$9 (US) elsewhere. See booth 1942.

Amersham's 1991 Life Science Products Catalogue is available free upon request (*Reader Service No. 117*). Over 2,000 **products for life science research** are listed. In addition to a separate A-Z product listing, more detailed information on the products and their applications is provided in colour-coded sections devoted to molecular biology, protein labelling and detection, immunological reagents, biomedical research assays, and structural formulae. Products are indexed both alphabetically and numerically by product code for ease of use. See booth 1614.

Assorted reagents

NycoPrep Mixer, a new separation medium from Nycomed, provides users with a one-step method for the **isolation of human mononuclear cells** (*Reader Service No. 118*). To isolate mononuclear cells, undiluted whole blood is mixed with NycoPrep. No layering of the sample is necessary, says Nycomed. After centrifugation at 1,500 g for 30 minutes at 20°C, the mononuclear cells in the supernatant are removed with a pipette. Lymphocytes can be isolated in greater than 90 per cent yield. NycoPrep Mixer is sold as a sterile, pyrogen-tested solution in 10 × 100-ml and 6 × 500-ml quantities for \$160 (US) and \$360 (US), respectively. See booth 1948.

Hunter's TiterMax is the new **immunoadjuvant** from CytRx, which is intended as a replacement for Freund's Complete Adjuvant (FCA) (*Reader Service No. 119*). CytRx has combined modern emulsion technology with synthetic copolymer adjuvants to produce an adjuvant that is designed to induce high antibody titres with minimal toxicity. TiterMax contains squalene, the new copolymer adjuvant (CRL89-41), a microparticulate stabilizer and an emulsifier. It is effective with antigens such as polysaccharides or detergent-solubilized proteins, which 'break' FCA emulsions, says CytRx. High antibody titres can be produced with a small volume in a single injection, and without the side effects of granulomas, adjuvant arthritis and chronic abscesses that are sometimes associated with FCA, says the company. One vial of TiterMax costs \$65 (US) and contains enough adjuvant to inject 10 rabbits or 10 mice. CytRx will be in booth 600. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. To obtain further details, use the reader service card bound inside the journal. Prices quoted are sometimes nominal, and apply only within the country indicated.



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